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CYTOLOGICAL CHANGES IN THE LIVER IN RIFT VALLEY FEVER, WITH SPECIAL REFERENCE TO THE NUCLEAR INCLUSIONS.

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THE generalized necrosis of the liver that is produced in lambs and in many small rodents by the virus of Rift Valley fever is of so acute a character that death not infrequently occurs in from 24 to 48 hours after injection of the virus. The necrosis is thus comparable in its rapidity to that produced by the action of certain chemical poisons rather than to that caused by other infections, for even in experimental yellow fever, where also there is rapid and extensive liver necrosis, death rarely takes place until the fourth or fifth day after inoculation. It therefore appeared to be of interest to compare the cytological changes produced in the liver by Rift Valley fever with those occasioned by acute chemical poisons and by the virus of yellow fever.

In the case of yellow fever an exhaustive examination of the cytological changes produced in the livers both of men and of experimentally infected *rhesus* monkeys had been carried out by Cowdry and Kitchen (1930), whose survey has been taken as a basis for the observations here recorded. Unfortunately the action of the two viruses in *rhesus* monkeys is somewhat dissimilar, for whereas yellow fever produces an extensive necrosis of the liver and is almost invariably fatal, Rift Valley fever produces only localized foci of necrosis and is never fatal. Therefore, although the cytological changes produced by the two viruses in the livers of *rhesus* monkeys were carefully compared, a more extensive series of observations was made on the livers of mice, in which species the whole process of a generalized necrosis can readily be followed from the time of inoculation to the fatal termination. At the same time, the lesions in sheep, goats, rats, squirrels, hamsters, voles and wood mice were also studied.

The cytological appearances investigated comprise both cytoplasmic and nuclear changes.

In the case of yellow fever the liver necrosis is as a rule primarily mid-zonal, while the line of demarcation between necrotic and non-necrotic areas is more or less distinct.

A. CYTOPLASMIC CHANGES.

In Rift Valley fever, on the other hand, the first stages of necrosis in all susceptible species are definitely focal. The foci may be found in any part of the liver lobule, though there does appear, at any rate in monkeys, to be a slight tendency for a majority of the lesions to be situated in the mid-zonal area of the lobule. Thus, of 50 foci counted in the livers of *rhesus* monkeys, 34 were mid-zonal, 10 periportal, and 6 central. In lambs, mice, rats and other small rodents, however, the foci extend so rapidly that in a very few hours the whole of the liver-tissue is involved. When the livers of mice are examined 10 to 12 hours after inoculation with 0·2 c.c. of a 1 in 10 dilution of infected mouse blood in citrated saline, it is found that necrosis may involve four or five contiguous liver-cells, or occasionally only a single cell. A few polymorphonuclear leucocytes may be seen in the sinuses. At 24 hours after inoculation the necrotic process involves roughly 50 per cent. of the liver-cells, while at 48 hours there is considerable difficulty in finding any healthy cells. In *rhesus* monkeys, on the other hand, the foci never become generalized. At the edge of the necrotic foci, more especially in sheep, and to a lesser extent in monkeys, there may be seen a form of *hyaline necrosis*, which leads to the formation of round hyaline bodies in the cell protoplasm. These hyaline bodies have a special affinity for eosin ; usually there is only one to a cell, the clear-cut margin of the body being frequently separated by a narrow empty space from the rest of the cell. Eventually the bodies are extruded, or in some cases pinched off from the remaining cytoplasm. In mice, while a few hyaline bodies may be seen at an early stage within the cell cytoplasm, it is commoner to find the whole cell becoming rounded and hyaline, with an increased affinity for eosin. In some cases the cell cytoplasm separates off from the cell membrane, which can be detected as a faint outline of the normal polygonal cell. Within the hyalinized cytoplasm some remains of the nucleus may still exist, though more commonly the nucleus has completely disappeared. At a late stage the affinity for eosin is not infrequently lost.

In the case of yellow fever, the formation of hyaline bodies, as pointed out by Cowdry and Kitchen (1930), is more common in man than in monkeys, in which animals the disease runs a very rapid course. Similarly in Rift Valley fever the hyaline bodies are common in the more resistant sheep and goat, rare in the highly susceptible mouse. Possibly, therefore, their presence in the cytoplasm may be indicative of the fact that only a localized portion of the cytoplasm has become necrotic—a portion which the still living cell attempts to extrude.

Two other generalized changes which are not uncommon in yellow fever may here be mentioned—fatty degeneration and pigmentation. In Rift Valley fever, both in monkeys and rodents, the occurrence of extensive *fatty degeneration* is rare. Occasionally in the livers of monkeys some degree of fatty infiltration may be seen in the periportal zone of the lobule. Similarly *pigmentation* of the liver-cells has not been seen in rodents, and only to a very slight extent in one *rhesus* monkey infected with Rift Valley fever. In yellow fever also pigmentation of the liver-cells is rare in monkeys, though not uncommon in man.

In order to throw further light on the cytoplasmic changes produced in the liver-cells by the virus of Rift Valley fever, observations were made on the changes produced in mitochondria and in the Golgi apparatus.

(1) *Mitochondria.*

It is now generally recognized that of the various cellular elements, the mitochondria are the first to undergo changes when the cell is exposed to toxic influences. By an examination of the mitochondrial changes in the various cells of an organ it is therefore possible to gain some idea of the course of an intoxication. In the case of the liver, special attention has been directed to the mitochondrial changes by the Lyons school of pathologists, and as a result of the work of Noël (1922), it has been found that under physiological conditions the liver lobule is divisible into three mitochondrial zones: (1) a periportal zone in which the cells, filled with granular mitochondria, are in a state of constant physiological activity, (2) a central zone, in which the cells have filamentous mitochondria—the zone of constant repose—and (3) an intermediate zone, in which the condition of the mitochondria varies with the state of the organ. During active digestion the mitochondria are granular, during repose filamentous.

The reaction of the mitochondria of the liver-cells to toxic agents, such as lead, manganese, copper, colloidal silica, amyl alcohol or anaphylactic shock is similar to that occurring during physiological activity. As shown by the work of Noël and Rosier (1924), Martin (1927), and Albot (1931), the first changes noted as a result of the action of these poisons are dilatation of the periportal capillaries, and a gradual disappearance of the mitochondria in the cells abutting on the portal spaces; later the mitochondria become granular in the cells, both of the intermediate and central zones. So far the mitochondrial changes in the liver caused by acute infections, as distinct from those due to the action of toxins, do not appear to have received any great attention.

In order to study the changes produced in the mitochondria of the liver by the virus of Rift Valley fever, mice were killed 6, 12, 24, 36 and 48 hours after subcutaneous inoculation with 0·2 c.c. of 1 in 10 mouse blood in citrated saline. The majority of mice receiving such a dose are usually moribund within 48 hours, though when smaller infecting doses are employed death may be delayed till the fifth or sixth day after inoculation. Portions of liver-tissue were fixed by the Schridde technique and stained with iron-hæmatoxylin and acid fuchsin. Six hours after inoculation the mitochondria in the liver-cells appeared normal, and the three zones in the lobule could be readily distinguished. Twelve hours after inoculation the filamentous mitochondria in some of the cells in the central zones had become granular, while in the periportal zones there was commencing chondriolysis, a progressive transformation of the mitochondria into small vacuoles, which eventually merge with the surrounding cytoplasm. In some cells a few large granular masses were all that remain. Twenty-four hours after inoculation the process had definitely progressed. While some cells still contained a few granular mitochondria placed close round the nucleus, the majority had entirely lost their mitochondria, the cytoplasm having become clear except for one or two dense granular masses. Forty-eight hours after inoculation the whole structure of

the liver lobule had been lost ; isolated rounded cells or even portions of cells were distributed irregularly ; the nuclei had broken down by rhexis, and the cytoplasm of the liver-cells, not infrequently vacuolated, merely contained a few irregular masses, some the last remnants of mitochondria, others derived from the nuclear chromatin.

The initial stage of the intoxication is thus similar in character to that produced by chemical poisons, where also lysis of the mitochondria is first seen in the cells of the periportal region.

(2) *Golgi Apparatus.*

The effect of virus infections on the Golgi apparatus has been comparatively neglected. Del Rio-Hortega (1914) described an initial hypertrophy followed by disintegration in the Golgi apparatus of the nerve-cells in a case of paralytic rabies. Ludford and Findlay (1926) in the epidermal cells of the chick infected with fowl-pox observed a similar sequence of events, hypertrophy being followed by breaking up of the Golgi apparatus into small granules. Ludford (1927) showed that the Golgi bodies are intimately related to the development of vaccinia lesions in the cornea of the rat, while Findlay (1929) figured a very marked hypertrophy of the Golgi apparatus in the connective-tissue cells of the rabbit infected with the *Virus myxomatosum*. In this case the hypertrophy was not followed by a stage of disintegration.

The Golgi apparatus in the liver-cells was studied by the Mann-Kopsch method, preparations being examined both after staining with neutral red and in the unstained condition. Mice were killed 6, 12, 24, 36 and 48 hours after inoculation as in the study of mitochondria. For the first 12 hours the Golgi apparatus only varied within normal limits. Later there was a definite hypertrophy in certain cells, with the formation of large granular masses, while in others there were only present a few osmophil granules. At 48 hours almost all trace of Golgi apparatus had disappeared from the liver-cells.

B. NUCLEAR CHANGES.

The most important changes caused by the virus of Rift Valley fever in the nuclei of the liver-cells are the margination of the chromatin on the nuclear membrane, and the appearance of acidophilic material in the nucleoplasm.

Intranuclear Inclusions.

To accumulations of acidophilic material in the nucleus associated with a number of virus infections it has been customary to apply the term " intranuclear inclusions ". In some ways this is perhaps unfortunate, since it suggests that the virus is actually contained within the inclusion body (as is the case in such virus diseases as fowl-pox, vaccinia and molluscum contagiosum). In the case of the intranuclear bodies associated with virus infections there is as yet no direct evidence that they are made up of or even contain the actual virus particles. They may, in fact, be due merely to some degenerative process in the nuclear material. In the present communication, therefore,

the term "intranuclear inclusion" is used without any implication that it denotes the actual presence of the virus within the nucleus.

The acidophilic intranuclear inclusions associated with Rift Valley fever were first described by Daubney, Hudson and Garnham (1931) in the liver-cells of sheep. Findlay (1932, *b*) confirmed the occurrence of these intranuclear inclusions in sheep and found them also in the livers of goats and *rhesus* monkeys, as well as in a number of small rodents (mice, rats, field voles, wood voles, grey squirrels and golden hamsters), which are susceptible to the infection.

The intranuclear inclusions in Rift Valley fever are restricted to the parenchymatous cells of the liver, and are found neither in the Kupfer cells, the epithelium of the bile-ducts, nor in the cells of other organs. In this respect, therefore, the virus of Rift Valley fever resembles the viscerotropic strain of yellow fever virus, while it differs from such viruses as those of herpes, Pacheco's parrot disease or Virus III infection, which are capable of giving rise to acidophilic intranuclear inclusions in the cells of a large number of different organs.

In the case of goats and *rhesus* monkeys infected with Rift Valley fever the hepatic lesions are localized in character, and the intranuclear inclusions are found only in hepatic cells at the periphery of the necrotic areas. In lambs and in small rodents in which the necrosis is more generalized, however, the inclusions are to be found, at some stage of the disease, within 80 to 90 per cent. of the liver-cells. The inclusions appear to be formed within the nucleoplasm of the liver-cells. As in the case of yellow fever, they may be seen when the liver-cells of the mouse or rat are examined in the living condition in isotonic media with direct illumination. As pointed out by Chambers (1924), the interkinetic nucleus in every normal living metazoan cell, when viewed either by direct illumination or with dark field equipment, is optically homogeneous, and possesses no visible structure except for one or more nucleoli and a delicate investing membrane. In the nuclei of the liver-cells of the mouse infected with Rift Valley fever and viewed by direct illumination when suspended in physiological saline, there are seen, in addition to one or more nucleoli, small irregular clumps of a definitely particulate character. These clumps are first visible some 12 hours after inoculation, and are present in from 80 to 90 per cent. of cells 24 to 36 hours after inoculation. Shortly before death, when many nuclei have broken down and disappeared, they are less numerous. As in the case of yellow fever, their examination in a fresh state is facilitated by the necrotic condition of the liver substance, as a result of which single cells are easily separated off by very slight mechanical injury.

When viewed in the fresh condition the intranuclear inclusions may be tinted by a number of vital dyes such as dilute water-soluble eosin, methylene blue, neutral red or phloxine red. They are not coloured by Janus green B, nor are they blackened by osmic acid. They are not pigmented in any way, though their refractive index differs slightly from that of the nucleoplasm in which they lie: they are not doubly refractile when viewed by polarized light.

The inclusions disappear if 0·1 per cent. acetic acid or 1 per cent. ammonia is allowed to run underneath the cover-glass, while if by pressure on the cover-glass the cells and nuclei are ruptured, the inclusion particles, when they escape into the surrounding medium, retain their size and shape apparently unaltered.

The fact that the intranuclear inclusions are visible in fresh liver-cells is strong evidence in favour of the view that these inclusions are not artefacts.

The inclusions withstand the action of all the commoner fixatives, such as Bouin's fluid, Zenker with and without acetic acid, formalin-Zenker, Regaud's mixture, sublimate acetic, Carnoy's fluid, 95 per cent. alcohol, and Bensley's acetic osmic-bichromate mixture. The tinctorial properties of the inclusions were studied as a routine in material fixed in Zenker's fluid containing the usual 5 per cent. of acetic acid. Before discussing their staining reactions, the development of the inclusions in relation to other nuclear changes will be briefly described as it is seen in sections stained with phloxine and methylene-blue after fixation in Zenker's fluid.

The structure of the nucleus in the normal liver-cell of the *rhesus* monkey has been described by Cowdry and Kitchen (1930). The nucleoli are normally amphoteric, consisting of a more or less acidophilic core, which is plastered or impregnated with basophilic material. The nucleoplasm in which the nucleoli are suspended contains traces of a network, as well as a host of irregular masses and spherules. This material is, as a rule, coloured blue by most of the basic stains, but in some cases may take on an acidophilic tint with eosin or phloxine. The development of the intranuclear inclusions in the liver-cells of the *rhesus* monkey is shown in Plate I, figs. 1–6. The earliest change is the appearance of one or more acidophilic granules in the area between the nucleolus and the nuclear membrane. As the inclusions increase in size the background of the nucleoplasm appears to become clearer, owing to a disappearance of the irregular spheres and vacuoles which are normally present in the healthy nucleus. As the inclusions increase in size also the spaces between them shrink and the nuclear membrane loses its smooth outline, becoming indented and irregular (Plate I, figs. 3 and 4). At the same time there is a gradual heaping-up of basophilic material on the inner side of the nuclear margin, and in some cases, as in Plate I, fig. 5, the nucleolus becomes definitely more acidophilic. As in the nucleus itself, the outer rim of the nucleolus remains more basophilic than the centre. Cowdry and Kitchen (1930), in describing the very similar changes coincident with the formation of the intranuclear inclusions in yellow fever, liken this separation of acidophilic and basophilic material to the transportation of differently charged material to the opposite poles of an electric field.

While a definite nucleolus may be present until a large acidophilic mass is visible in the nucleoplasm, more commonly there remain in the nucleoplasm only a few small basophilic masses (Plate I, figs. 2, 3 and 6) entirely surrounded by acidophilic granules. In some cases there may be little or no basophilic material attached to the nuclear membrane, which eventually becomes indistinguishable from the surrounding cytoplasm.

The development of the inclusions in other animals is very similar to that described in the *rhesus* monkey. In mice (Plate I, figs. 13–18) the separation of basophilic and acidophilic material is well seen, and appears to be rather more precise than in the monkey, for it is not so common to find basophilic droplets remaining in the centre of the acidophilic masses. At the same time the acidophilic material is separated from the basichromatin attached to the nuclear membrane by a clear area. In the mouse also it has been possible to trace what appears to be the gradual migration of the nucleolus towards the

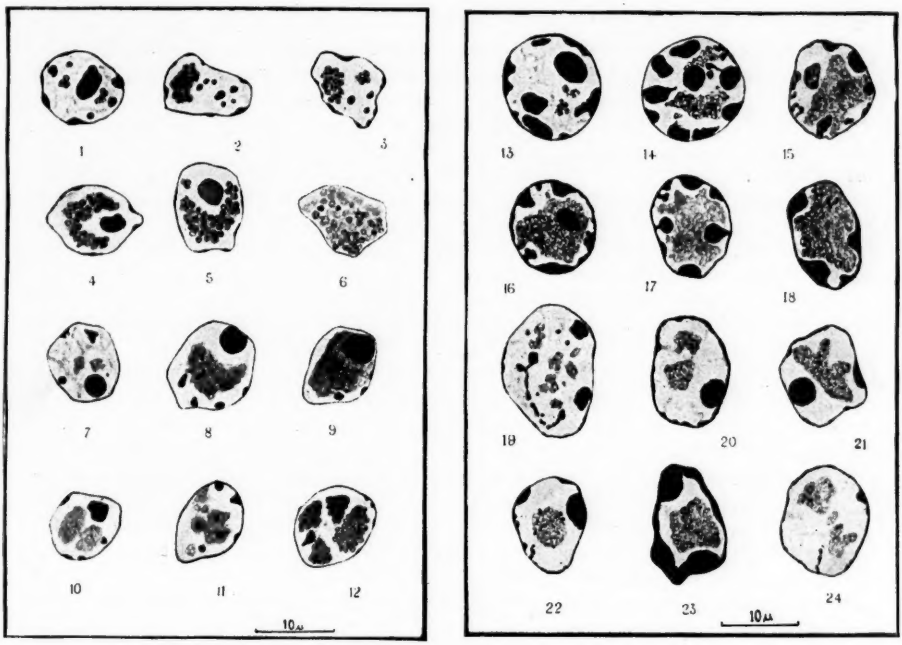

PLATE I.

Camera lucida drawings of the nuclei of liver-cells from animals suffering from Rift Valley fever tissues fixed in Zenker's fluid and stained with methylene-blue and phloxine red.

FIGS. 1–6 illustrate Rift Valley fever inclusions in the liver-cells of *Macacus rhesus* monkeys.

FIGS. 7–12 illustrate yellow fever inclusions in the liver-cells of the same species for comparison.

FIGS. 13–18 illustrate Rift Valley fever inclusions in the liver-cells of the mouse; FIGS. 19–24 in the liver-cells of the rat.

Findlay.

nucleolar membrane, with which it eventually becomes fused. Finally, when large basophilic masses have become attached to the nuclear membrane this gives way; the acidophilic inclusion is lost to view in the cytoplasm of the cell, while the basophilic material separates into droplets which pass to the periphery of the cell. Destruction of the cell by rhexis is thus much more common than the so-called process of lysis, in which the wholly acidophilic nucleus merges into the acidophilic cytoplasm.

In order to study the genesis of the intranuclear inclusions in the mouse, it is therefore essential to examine the liver before necrosis has become general. It is then possible to determine the presence of acidophilic material in from 80 to 90 per cent. of the liver-cells, many of which are actively dividing, so that cells with two nuclei, each of which contains acidophilic material, are by no means rare.

The question whether cells in which intranuclear inclusions have once developed can recover is of some interest, and cannot at the moment be answered conclusively. It may, however, be noted that in the liver of the goat the inclusions which are present in only a few cells at the edge of necrotic foci are small, and rather more condensed than in the other animals. Clinically the goat on which these observations were made was on its way to recovery when killed.

So far chemical tests have thrown little or no light on the composition of the intranuclear inclusions in Rift Valley fever. They are not blackened by osmic acid, nor in frozen sections do they stain with sudan III, Scharlach R or Nile blue sulphate. They do not give a positive reaction for chromatin by the Feulgen technique, nor any evidence of masked iron by the Bensley-Macallum test. The staining reactions of the intra-nuclear inclusions are shown in the table, where the tinctorial properties of the inclusions caused by Rift Valley fever in the livers of *rhesus* monkeys are compared with those of the inclusions produced in the liver-cells by the virus of yellow fever. The colours were determined by comparison with Ridgway's ' Color Standards and Color Nomenclature ' (1912). To ensure a reliable comparison of the inclusions in the two diseases, sections of the livers of *rhesus* monkeys suffering from Rift Valley fever and yellow fever were mounted and stained on the same slide.

It should be noted that the staining reactions of the inclusions do not change as the acidophilic masses increase in size—a point in which they differ from the inclusions in the liver-cells associated with the Clacton disease of mice (Findlay, 1932, *a*).

Apart from the intranuclear inclusions and the rearrangement of the nuclear chromatin which is related to their development, other nuclear changes are but rarely met with. Nuclear budding, which is characterized by the nucleus sending out buds or extensions, which break away from the original nucleus, was not seen in any of the animals affected with Rift Valley fever. Cowdry and Kitchen (1930) noted its occurrence in human cases of yellow fever, but not in monkeys experimentally infected with the same disease; in addition "empty" nuclei (that is, hypertrophied nuclei with margination of the chromatin, but no central acidophilic inclusions) were not observed either in monkeys or mice with Rift Valley fever. Vesiculated nuclei were also uniformly absent in the livers of animals infected with Rift Valley fever.

Staining Reactions of the Intranuclear Inclusions in the Liver-cells of Rhesus *Monkeys Infected with Rift Valley Fever and Yellow Fever.*

(After fixation in Zenker's fluid with 5 per cent. acetic acid.)

	Stain.	Rift Valley fever.	Yellow fever.
1.	Iron hæmatoxylin and eosin.	Eosin pink to grenadine pink.	Eosin pink to grenadine pink.
2.	Giemsa.	Light coral red to coral red.	Light coral red to coral red.
3.	Eosin and methylene-blue.	Eosin pink.	Eosin pink.
4.	Phloxine and methylene-blue.	Rose dorée.	Rose dorée; spectrum red.
5.	Resorcin fuchsin.	Spectrum red.	Spectrum red.
6.	Phosphotungstic acid hæmatoxylin.	Hay's russet.	Hay's russet to dark Indian red.
7.	Mallory's connective stain.	Spectrum red.	Spectrum red.
8.	Castaneda's stain.	Burn blue to pale forget-me-not blue.	Forget-me-not blue.
9.	Neutral red.	—	—
10.	Neutral gentian violet.	Deep purple vinaceous.	Deep purple vinaceous.

THE INTRANUCLEAR INCLUSIONS OF RIFT VALLEY FEVER AS COMPARED WITH THOSE PRODUCED BY OTHER VIRUSES IN THE LIVER.

From what has been previously said, it is obvious that morphologically and tinctorially the inclusions produced by the viruses of Rift Valley fever and yellow fever are very similar. The main difference is that whereas the inclusions of yellow fever are restricted to certain species of monkey and to man, those of Rift Valley fever can be found in a wide variety of susceptible species.

Morphologically also the inclusions of Rift Valley fever are in most animals as a rule, rather more delicate or tenuous in appearance than those of yellow fever, while their distribution in monkeys also differs. In monkeys dying of yellow fever 80 to 90 per cent. of liver-cells have characteristic intranuclear inclusions. In monkeys with Rift Valley fever, on the other hand, the inclusions are found only in cells at the periphery of the focal lesions, while in lambs and in many rodents actually dying from the disease, the presence of intranuclear inclusions is often difficult to determine owing to the fact that degenerative changes in the cells are so far advanced.

Intranuclear inclusions in the liver-cells, however, have now been described in a number of virus diseases in addition to yellow fever and Rift Valley fever. Thus Goodpasture (1925) and Cowdry and Kitchen (1930) have shown that herpes produces intranuclear changes in the liver-cells of monkeys. Pacheco's parrot virus also gives rise to very characteristic intranuclear inclusions in the liver (Pacheco and Bier, 1930; Rivers and Schwentker, 1932), as does the Clacton disease of mice described by Findlay (1932, *a*).

In the case of herpes in monkeys, Cowdry and Kitchen (1930) described not only slight tinctorial differences between the herpes inclusions and those caused by yellow fever, but also certain morphological appearances which are characteristic of the two viruses. When herpetic inclusions begin to form in a localized part of the nucleoplasm, they are generally limited by a halo of unstainable substance in which there is no basophilic chromatin. Haloes of this kind are not so readily detected in yellow fever. In the case of yellow

fever the amphinucleolus also generally maintains its central position in the nucleus until after the inclusions have become well developed, while in herpes the basophilic component is more quickly split off from the amphinucleolus and the remaining substances become marginated on the nuclear membrane with the rest of the nuclear chromatin. Central solitary nucleoli associated with moderately developed inclusions are therefore rare in herpes, but common in yellow fever. In Rift Valley fever in monkeys also they are not uncommon.

The morphology of the individual particles is much more uniform in yellow fever than in herpes; this is also the case in Rift Valley fever. Finally in herpes the inclusions are not broken up into clumps as in yellow fever and Rift Valley fever, but form a single large inclusion mass.

On the other hand, there is a similarity between herpes and Rift Valley fever in the liver of the monkey, since in herpes also comparatively few cells in limited foci are affected, while the general framework of the liver remains intact.

In Pacheco's parrot disease the intranuclear inclusions are not confined to the liver-cells, but may be found in a variety of organs, though when fully developed they are not unlike those caused by the viruses of yellow fever and Rift Valley fever. The inclusions begin in the nucleoplasm in the form of a delicate acidophilic granular mass usually in close proximity to the nucleolus or, when the cell contains two nucleoli, in the region between them. As the inclusions grow in size the nucleoli retreat towards the nuclear membrane, with which they eventually blend. Chromatin particles are rarely seen in the interior of the acidophilic masses. On the other hand, the inclusions are almost always separated from the nuclear membrane by a clear area equal in width to about half the diameter of the inclusions. The outline of the inclusions is as a rule definite and precise, sometimes quite smooth, at other times serrated. In very many cells there are no chromatin particles adherent to the nuclear membrane, nor does the membrane rupture in the way that occurs in Rift Valley fever where chromatin particles are scattered in the cytoplasm. The nucleus thus disappears by lysis rather than by rhexis.

For slides illustrating the appearance of the liver lesions in this virus disease of parrots I am indebted to Dr. T. M. Rivers, of the Rockefeller Institute of Medical Research, New York.

The appearance of the intranuclear bodies described and figured by Findlay (1932, *a*) in the livers of a certain strain of "Clacton" mice differs considerably from that of the intranuclear inclusions already described, for in this infection of low pathogenicity the bodies are bounded by a definite basophilic membrane, and, when small, are only slightly acidophilic. They do not arise in the nucleoplasm. The morphology and the staining properties of the bodies suggest that they may be due rather to an hypertrophy of the nucleoli.

The five viruses discussed thus differ among themselves not only as regards the morphology of their intranuclear inclusions, but also as regards their power to produce similar changes in the cells of organs other than the liver. They differ also to a lesser extent as regards their tinctorial properties.

As controls for the occurrence of acidophilic intranuclear inclusions there were examined normal livers of *rhesus* monkeys, rats and mice, and the livers of rats and mice poisoned by carbon tetrachloride, arsphenamine, manganese

chloride and tar. In none of these animals were there seen acidophilic intranuclear inclusions similar to those met with in yellow fever or Rift Valley fever. Occasionally small masses of acidophilic material were detected in the nucleoplasm both of normal animals and of those with livers damaged by poisons, but in no livers was there any evidence of a progressive increase in the size of the acidophilic masses such as is seen in Rift Valley fever.

However the virus inclusions are produced, it is obvious that they are not readily formed by the action of well-known chemical toxins on the liver-cells.

DISCUSSION.

From the foregoing description of the lesions in the liver in Rift Valley fever, it is obvious that chief interest attaches to the formation of the intranuclear inclusions. Four possibilities as to the origin of the inclusions must be considered: That they are due to (1) artefacts, (2) masses of virus, (3) degeneration of the nucleoplasm, (4) the presence of virus and an associated degeneration of the nucleoplasm.

The first possibility can now hardly be maintained; since the inclusions can be seen in fresh preparations they cannot be due to the action of fixatives on the liver-cells. Evidence in favour of the second possibility is far from strong. The view that in herpes the intranuclear bodies are essentially masses of virus was first maintained by Goodpasture and Teague (1923), who laid special stress on the small masses of basophilic material which, they claimed, may be interspersed with the acidophilic particles. As pointed out by Cowdry and Kitchen (1930), however, the acidophilic character of the bodies in herpes and yellow fever, the absence of thymus nucleic acid and the negative reaction for iron all contra-indicate living particles. These characteristics apply also to the inclusions of Rift Valley fever.

It must, however, be remembered that at present we know very little, if anything, of the chemical composition of any virus.

More recently also evidence has been accumulating as to the size of certain of the viruses associated with intranuclear inclusions. Thus Elford, Perdrau and Smith (1933) have found that the virus of herpes has a probable size of from 0·1 to 0·15μ; as a result it should be theoretically possible to photograph this virus by means of ultra-violet light. Broom and Findlay (1933), on the other hand, have shown that the virus of Rift Valley fever is definitely smaller, and has a probable size of from 23 to 35 mμ, while unpublished experiments indicate somewhat smaller dimensions for the virus of yellow fever. These viruses are therefore below the limit of microscopic visibility even with ultra-violet light.

The view that the acidophilic material is a product of degeneration has much to commend it, and has recently gained added force from the experiments of Davenport, Ranson and Terwilliger (1931), who immersed various tissues in hypertonic saline solution. As a result, the chromatin shrank from the nuclear membrane, while acidophilic material appeared in the nucleoplasm of the cells. Such changes were obtained in the ganglion cells of the turtle, in the spinal ganglia cells of the dog, and in one specimen of cat kidney. Attempts to originate similar changes in liver, adrenals and testes were unsuccessful.

The possibility that the nuclear changes associated with certain virus diseases are due to a loss of water from the nucleus is one which is of considerable interest. Both in Rift Valley fever and in yellow fever, with the growth of the inclusions, the nuclear membrane becomes crumpled and shrunken as if fluid had been lost by osmosis.

The fourth possibility, that the intranuclear inclusions consist of virus coated by precipitated nucleoplasm, is one which at the present time cannot be supported by any direct evidence, except on the analogy of such structures as the Bollinger bodies in fowl-pox, which appear to be composed of virus particles surrounded by a fatty envelope derived from the cell cytoplasm. The inclusions of Rift Valley fever, on the other hand, cannot be resolved into elementary bodies, nor is there any indication that they are composed of two sorts of substance.

The work of Scott (1930) on the micro-incineration of the cells in the submaxillary glands of guinea-pigs containing virus inclusions, and the more recent micro-incineration studies of Cowdry (1933) on yellow fever inclusions in the liver-cells of *rhesus* monkeys, show that the inclusions do not possess sufficient mineral content to leave a recognizable ash. This is also suggestive evidence in favour of the view that the intranuclear inclusions produced by these viruses are not of an organismal nature.

CONCLUSIONS.

1. Intranuclear inclusions have been found in the liver-cells of the sheep, goat, *rhesus* monkey, marmoset, rat, mouse, field vole, wood vole, golden hamster and grey squirrel infected with Rift Valley fever.

2. The inclusions develop in the nucleoplasm of the liver-cells and are visible in fresh preparations.

3. The inclusions do not give a reaction for fats, thymonucleic acid or masked iron.

4. The development of the acidophilic intranuclear inclusions coincides with the margination of the nuclear chromatin on the nuclear membrane, which eventually ruptures.

5. The morphology and tinctorial properties of the inclusions are described and compared with those associated with other viruses which give rise to inclusions in the nuclei of the cells of the liver.

6. The lysis of mitochondria and the disruption of the Golgi apparatus in the liver-cells in Rift Valley fever are also described.

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VARIATION IN AGGLUTINATION OF STOCK CULTURES OF MENINGOCOCCI.

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THERE is a considerable amount of evidence which does not support Murray's original statement that the fixity of the four English types of meningococci appears to be absolute (Murray, 1929), as he himself later indicated (1932).

Walker (1918) concluded from the evidence available at the time that the fixity of type was not proven, and that "the meningococcal types differentiated in relation to the rabbit do not appear to represent independent entities".

Kristensen and Moltke (1926), after a long series of agglutination and absorption tests on English and Danish strains of meningococci (including all the strains present at that time in the "National Collection of Type Cultures" at the Lister Institute), failed to discover any stable system of typing meningococci, and concluded that no generally applicable division of meningococci into groups was then known.

Among the organisms examined by these authors was Littledale (type I, No. 668), which is under discussion in the present paper. On testing suspensions of this organism with sera prepared from another type I meningococcus (Howse, 214) they found that no agglutination occurred, whereas in two different type III sera the suspensions were strongly agglutinated. They

also found that suspensions of Littledale absorbed one type III serum just as completely as the homologous strain, whereas they did not absorb another serum which had been prepared from a type I strain.

Branham (1932) has shown that in America only a few meningococci isolated in recent epidemics appear type-specific, and although she succeeded in placing most of the strains she examined into one or other of Gordon's four types, she found the task of typing the organisms greatly complicated by antigenic instability and a lack of strict group specificity. She made a comparison between the living organisms of the original strains of Gordon and the same strains kept since 1917 in the dry state, and showed that the living strains had spread antigenically, one of the original type I strains, for example, behaving like type III, although the dried antigen made years before from the same strain still gave a narrow type I reaction.

American type sera and suspensions (kindly supplied by Miss Branham) have been compared with the type-sera and suspensions in use at the Standards Laboratories, Oxford, and have been found to show overlapping of all four types, especially types I and III. For instance, when typed by agglutination with American type-sera, Standards strain Littledale (type I) reacts as a type III organism, with cross agglutination in serum type I, and Standards strain Whitehouse (type III) behaves as a type I organism, with broad overlapping into types III and II. These results with the American sera will be seen to parallel those obtained by Kristensen and Moltke, quoted above, who found that Littledale was not agglutinated by their arbitrary type I serum, although it was strongly agglutinated by two different type III sera.

It is evident from the above that the antigenic reactions of cultures of meningococci are not always stable, and the work described in this paper is an attempt to discover where this instability lies.

So far it has not been possible to grow meningococci from single cells, and investigation of the properties of single colonies derived from the same parent culture (on the lines of the work of Andrewes [1922] on the Salmonellas) has been confined mainly to the work of Kondo (1925), who has shown that there may be a wide variation in the agglutinability of single colonies when compared with that of the parent strain, and that easily agglutinable colonies may sometimes be obtained from cultures which are themselves agglutinated only with difficulty.

In the following paper, investigation on these lines has been continued on single colonies derived from Standards Laboratories stock type cultures Littledale (type I) and Whitehouse (type III).

EXPERIMENTAL METHODS.

In outline, the experiments consisted first in obtaining a " pure " growth of each of the type meningococci (derived from a succession of single colonies), then growing these strains in various ways on different media, and finally examining the reactions of single colonies selected from them.

The " pure " strains of both Littledale and Whitehouse were obtained by plating out each organism on egg six consecutive times, a single colony being

selected each time, subcultured on egg medium for 24 hours and then replated. The final (seventh) colonies selected in this manner were used, after 24 hours' growth on Dorset's egg medium, as the " pure " cultures of Littledale and Whitehouse in the following experiments.

Treatment of " Pure " Cultures of Littledale and Whitehouse.

The cultures of Littledale and Whitehouse prepared as above were subcultured on to large slopes of Dorset's egg medium and into tubes of broth. (pH 7·6).

For the next 10 weeks half the egg slopes of each organism were subcultured on to fresh egg slopes at weekly intervals. The rest of the egg slopes, together with the broth cultures, were stored untouched in the incubator at 37° C. for the same length of time.

At the end of 10 weeks' incubation the cultures were divided up in the following manner :

The egg slopes which had been subcultured weekly were numbered Littledale S and Whitehouse S respectively. The egg cultures which had not been subcultured were numbered Littledale O and Whitehouse O respectively, and the broth cultures were numbered Littledale B and Whitehouse B.

On subculture of all the above strains on to fresh egg slopes, growth appeared in all cases within 48 hours, and after a further 24 hours' growth on egg the organisms were plated out on to egg, and single colonies were selected from them after 24 hours' incubation.

In this manner 30 single colonies were obtained from Littledale S (numbered L. S 1–30) ; 18 from Littledale O (numbered L. O 1–18) ; 17 from Littledale B (numbered L. B 1–17).

From the Whitehouse strains, 27 colonies were obtained from Whitehouse S (numbered W. S 1–27), and 16 from the B culture (numbered W. B 1–16). The Whitehouse O culture was lost.

Preparation of Suspensions.

The single colonies derived as above were allowed to grow for 48 hours on egg and after subculture on to fresh egg slopes for a further 24 hours, suspensions in phenolized (0·5 per cent.) physiological saline were prepared from them and standardized after the method of Gordon (1915) to contain 2000 million organisms per c.c. of the suspension.

Similar suspensions were also prepared from the parent strains Littledale O, S and B and Whitehouse O, S and B.

These suspensions were then tested against sera prepared by weekly intravenous inoculation of rabbits with Standards Laboratories stock type suspensions, Littledale (type I), Myott (type II), Whitehouse (type III) and Hicks (type IV).

In all the agglutination tests which follow, Dreyer's macroscopic method was used over a period of 24 hours at a temperature of 55° C., and absorption of agglutinins was performed after the method of Gordon (1915).

RESULTS OF EXPERIMENTS.

Agglutination of Type Suspensions, Suspensions of Parent Strains and Single Colonies Derived from them.

Suspensions of type strains, parent strains and single colonies derived from them were tested against the type-sera with the following results, shown collectively in Table I.

It will be seen from Table I (col. 1) that the type-sera used in the experiments are antigenically broad rather then narrowly specific, especially types I and III.

The agglutination of the parent strains (Table I, cols. 2 and 3) does not disclose any great differences between them, except that Littledale O overlaps more than the other strains of Littledale into type III, but the study of the agglutinability of single colonies derived from the parent strains shows marked variations.

For instance, in serum 1 cross agglutination occurs at a dilution of 1/100 in 14 out of 16 colonies of Whitehouse B, and in only 10 out of 27 colonies of Whitehouse S (Table I, col. 6). A similar degree of cross-agglutination occurs in sera II and IV, but whereas all colonies of Whitehouse B agglutinate in all sera at a dilution of 1/50, 5 of the colonies of Whitehouse S fail to agglutinate in either sera II or IV.

The colonies derived from Whitehouse B, therefore, appear broader antigenically than those of Whitehouse S, although both these strains originated from the same Standards type-culture. This difference is presumably due to the different methods of cultivation adopted in the two cases.

A similar variation is seen on comparing the agglutinability of the single colonies of the three Littledale strains (Table I, col. 5). For example, in serum III, 17 out of 30 colonies of Littledale S agglutinate at a dilution of 1/250, which exceeds the end-point of agglutination of the Standards Littledale strain. Only two colonies of Littledale O agglutinate at this dilution in the same serum, while none of the colonies of Littledale B agglutinate in Serum III even at 1/100, at which dilution the Standards strain is readily agglutinable.

It thus appears that both Littledale S and O have broadened antigenically so as to overlap more than the Standards strain (from which they were derived) into the heterologous sera, especially serum III. On the other hand, the colonies of Littledale B do not exhibit similar broadening.

The Properties of Sera made from the Parent Strains.

Sera prepared by the intravenous inoculation of rabbits with suspensions of the parent strains were tested against suspensions of Standards type organisms, with the results shown in Table II.

From Table II it is seen that the sera prepared from suspensions of the three parent strains of Littledale, when tested against the Standards type suspensions, exhibit a much greater overlapping into type III than is shown by the agglutination of the parent suspensions themselves in type-sera (Table I, col. 2).

TABLE I.—*Agglutination of Type Suspensions, Parent Strains and Single Colonies derived from them.*

	Titres with Standards type suspensions.		Titres with parent cultures.						Serum dilutions.	Number of colonies showing end-point at various dilutions of sera. Strain.				
	I (L.)	III (W.)	L. O.	L. S.	L. B.	W. O.	W. S.	W. B.		L. O.	L. S.	L. B.	W. S.	W. B.
										Total colonies.				
										18.	30.	17.	27.	16.
Serum type I	500	100	500	500	500	100	100	100	100	—	—	—	10	14
									50	—	—	—	17	2
									25	—	—	—	0	0
									<25	—	—	—	0	0
Serum type II	50	100	50	50	50	50	50	50	100	13	27	0	10	14
									50	2	0	5	11	2
									25	0	2	0	0	0
									<25	3	1	12	6	0
Serum type III	100	500	250	100	50	500	500	500	250	2	17	0	—	—
									100	13	12	0	—	—
									50	3	1	17	—	—
									25	0	0	0	—	—
									<25	0	0	0	—	—
Serum type IV	25	50	50	50	25	50	100	25	100	0	0	0	10	14
									50	18	20	2	9	2
									25	0	7	0	3	0
									<25	0	3	15	5	0

TABLE II.—*Agglutination of Type Suspensions in Sera Prepared from Parent Strains.*

Suspensions.	Titre of sera for suspensions. Sera.					
	L. O.	L. S.	L. B.	W. O.	W. S.	W. B.
Type I	500	500	500	100	100	100
,, II	100	100	100	100	100	100
,, III	500	250	500	500	500	500
,, IV	100	100	250	100	50	100

For instance, sera prepared from Littledale O and B both agglutinate type-suspension III at a dilution of 1/500, and the serum from Littledale S agglutinates this suspension at 1/250, although Littledale O is the only Littledale parent strain agglutinating in type III serum at a dilution of more than 1/100 (Table I, col. 2).

On the other hand, Table II shows that sera prepared from Whitehouse strains overlap into all types at a dilution of 1/100, but do not show any of the great broadening seen in the Littledale sera.

Agglutination of Parent Strains in Homologous Sera.

The sera prepared from the Littledale and Whitehouse parent strains were tested against the suspensions of these strains with the following results: All the Whitehouse sera agglutinated the Whitehouse suspensions at a dilution of 1/500, and the Littledale suspensions at 1/100, except Littledale S, the suspension of which was not agglutinated at any dilution greater than 1/50.

All the Littledale sera agglutinated the Littledale suspensions at a dilution of 1/500. When tested against the Whitehouse suspensions, however, the titres of the Littledale sera varied as shown in Table III.

TABLE III.—*Agglutination of Parent Strains in Homologous Sera.*

Serum.	Titres with parent suspensions. Suspension.		
	W. O.	W. S.	W. B.
Littledale O	100	100	250
,, S	100	250	250
,, B	100	250	250

Absorption of Type-Sera with Parent Strains and Single Colonies.

Absorption of type-sera with parent strains of Whitehouse removed the type III agglutinins from the type III serum, and left the type I serum unaffected. Absorption of type-sera with parent strains of Littledale, however, produced unexpected results, in that type III serum was absorbed more completely than the homologous type I serum. This anomalous absorption of type III agglutinins by the type I parent suspensions will be referred to in more detail later.

Absorption of type-sera with single colonies derived from Whitehouse strains removed the agglutinins from the homologous type III serum and

did not affect type I serum. On the other hand, as in the case of the parent strains, absorption with single colonies derived from the Littledale strains removed more of the type III agglutinins than those of the homologous type I.

Table IV illustrates the effects of absorption of type-sera I and III with the parent strains of Littledale and Whitehouse, and with two typical single colony suspensions.

TABLE IV.—*Effects of Absorption with Parent Strains and Single Colonies.*

Serum.	Titre with homologous suspensions after absorption with—			
	Littledale (parent).	Whitehouse (parent).	L. O/13.	W. B/3.
Type I	125	>250	>250	>250
„ III	25	25	0	0

DISCUSSION.

The use of standard technique throughout the foregoing experiments makes the results comparable.

Investigation of the agglutinability of the parent strains of Littledale and Whitehouse in the broad type-sera does not reveal the great variation which is found to occur amongst single colonies derived from them.

Undisturbed growth in broth appears to have increased the agglutinability of both the Whitehouse strains more than frequent subculture on egg, whereas the Littledale strains have become more agglutinable after subculture on egg, and continuous growth in broth has lowered their general power of cross-agglutination (Table I).

Examination of the agglutinability of single colonies of the parent strains (Table I, cols. 5 and 6) shows considerable variation amongst the colonies themselves, compared with the parent strains, and although certain rough grouping of these colonies is possible, depending on the degree of cross-agglutination present, there is no sign of any fundamental distinction between the groups, such as is seen in the Salmonellas (Andrewes, 1922).

Testing the sera prepared from the parent strains against the homologous suspensions, and also the type suspensions, shows that the Littledale strains are broader than the Whitehouse strains (Tables II and III).

For example, none of the Whitehouse sera has a titre for Littledale suspensions greater than 100, whereas sera prepared from both Littledale B and S have a titre of 250 for both suspensions Whitehouse B and S. All the Littledale sera have a titre of 250 for strain Whitehouse B (which by agglutination in type-sera appears as the broadest Whitehouse strain) and for strain Whitehouse S (except serum derived from Littledale O).

The parent strain Littledale S, therefore, seems to have broadened more than Littledale O, which is the reverse of the result of agglutinating Standards type suspensions in the Littledale sera (Table II). It will be seen, however that in the agglutination of single colonies of Littledale O, broadening into type III has taken place in only 2 out of 18 colonies, whereas more than half the colonies of Littledale S are agglutinated in serum III at higher dilutions than the parent strain (Table I). The broadening changes occurring in

Littledale O, therefore, seem to be progressing more slowly than those in Littledale S.

Absorption of type-sera with suspensions of Whitehouse parent strains results in almost complete absorption of agglutinins from the homologous type III serum and a slight absorption from type I serum. Absorption with the parent strains of Littledale, however, produces unexpected results, as in every case more type III agglutinins are removed from serum III than type I agglutinins from the homologous serum I (Table IV).

Thus, it seems that the Littledale parent strains have developed antigens of type III which are capable of absorbing the type III serum almost completely, whereas their type I antigens have either altered from the original form, or else degenerated until they are incapable of completely absorbing the type I serum.

As far as absorption tests are concerned, therefore, these strains of Littledale behave more like type III than type I, although they are all derived from the same original type I Littledale strain.

Absorption of type-sera with suspensions of single colonies shows the same excessive absorption of type III agglutinins by the Littledale organisms, although the degree of absorption varies from colony to colony (see Table IV for tests with L. O/13).

On the other hand, absorption of the type-sera with suspensions of single colonies derived from the Whitehouse strains removes all the agglutinins from the type III serum, but leaves the type I serum unaffected.

SUMMARY.

Great variation in agglutination by " type " sera occurs amongst single colonies of stock meningococci.

Littledale (type I) strains show a tendency to overlap into type III to such an extent that these types appear to be approaching interchangeability

There is no evidence of a division of group and specific antigens among single colonies derived from stock type cultures.

The so-called types I and III of the meningococcus are in all probability not fixed types.

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ROUGH AND SMOOTH VARIANTS IN STOCK CULTURES OF MENINGOCOCCI.

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PETRIE (1932) has shown that the " smooth " variant of the pneumococcus, when grown as a stab culture on homologous immune-serum agar, develops a faint white opaque halo round the growth after about 24 hours' incubation, whereas the " rough " variant stabbed into the same medium produces different and fainter haloes.

He also found that fresh meningococcal cultures of types I and III produced similar haloes round stab growths on homologous serum agar, whereas old cultures of the same types produced either faint, imperfect haloes or none at all.

In the case of the Shiga dysentery bacillus, the addition of the purified bacterial polysaccharide to the homologous serum-agar medium produced clouding of the medium, and Petrie therefore considered that the haloes produced by organisms were probably due to the precipitation by the immune serum of some soluble substance, *e. g.* a carbohydrate, which diffused from the growing organism into the medium.

The investigations described in this paper were undertaken in the hope of finding variants of meningococci which produced no specific soluble substance, and therefore no haloes in the homologous serum-agar medium.

On the analogy of Petrie's work on the pneumococci, it was thought that variants of this kind might prove to possess " rough " characteristics.

Experiments performed on these lines have shown the existence of at least two forms of variant in the meningococci examined.

THE PRODUCTION OF HALOES ROUND MENINGOCOCCAL CULTURES.

Cultures of Littledale (type I, Standards Laboratories, Oxford) and Whitehouse (type III, Standards Laboratories), when stabbed into serum-agar medium prepared from immune horse-serum of mixed types I and III (kindly supplied by Dr. Petrie), developed haloes after incubation at 37° C. for 24 hours.

The haloes round the growths of Whitehouse were very similar to those described by Petrie, " forming a delicate annular opacity round the colony and best seen against a black background ". They did not appear to spread out from the growth, but were first seen and reached their maximum density about 2 mm. from the growth, gradually falling off in intensity near the culture. The haloes remained the same size for several days, and then either spread out

evenly and more rapidly than before or took the form of advancing concentric rings.

Stab inoculations of Littledale usually showed no halo until after 24 hours' incubation. The haloes were always close in against the edges of the growth and had the appearance of coming from beneath the culture. They were best seen by examining the medium obliquely. After 4 or 5 days they spread out into the medium, generally as faint concentric rings.

Faint haloes similar to those produced by Whitehouse were developed round stab inoculations of Littledale in homologous serum-agar medium containing 2 per cent. glycerin.

In homologous rabbit-serum agar Littledale and Whitehouse both failed to develop haloes. It was incidentally discovered that the addition of normal laked rabbit blood to the immune horse-serum agar produces a clear pink medium in which meningococci grow with great rapidity and haloes develop within 5 hours.

THE DETECTION OF VARIANTS.

As both Littledale and Whitehouse developed haloes in the immune horse-serum agar medium, single colonies were picked from both strains and inoculated as stabs on to plates of the medium in order to detect any differences in halo formation.

Of 16 single colonies selected from a 10-weeks old broth culture of Whitehouse, 12 developed haloes, some of which were strong and diffuse, others narrow and faint. Four of the colonies did not produce haloes even after repeated subculture on egg.

Colonies selected from Whitehouse after 10 weeks on a single tube of Dorset's egg-medium all developed haloes in the immune serum-agar, and of 27 colonies picked from a culture of Whitehouse which had been subcultured weekly on egg medium, all produced strong haloes except one (W. S/17).

Five out of 21 colonies selected from Littledale after incubation for 10 weeks in broth failed to develop haloes in the serum-agar medium.

It can be seen from the above results that the development of haloes in homologous immune-serum agar is not a constant feature of single colonies derived from mass cultures which themselves develop haloes. All the colonies mentioned above which failed in the first instance to develop haloes have since consistently failed to do so.

In order to determine whether this failure to produce haloes was a true characteristic of the growths, two single colonies were selected for further investigation, one (W. S/1) developing haloes, the other (W. S/17) not doing so.

These colonies were plated out on egg medium, and single colonies were selected from them after 24 hours' incubation and stabbed into plates of the immune-serum agar. All the single colonies from W. S/1 developed characteristic haloes within 24 hours, but those selected from W. S/17 showed none, even after several days' incubation.

Single colonies derived from other non-halo-producing colonies of Whitehouse and Littledale similarly failed to develop haloes in the serum-agar medium, whereas single colonies derived from halo-producing colonies were found to fall into two groups. In cases where the halo round the original colony was

diffuse and strong, derived colonies were usually all found to be halo-producers, but where the halo round the original colony was faint or irregular, the derived colonies were found to be mixed, some developing haloes, others not.

The single colonies derived from Whitehouse and Littledale were therefore found to be of three kinds, namely :

(*a*) Colonies producing haloes.

(*b*) Colonies not producing haloes.

(*c*) Colonies developing faint or irregular haloes.

Of these kinds of colonies, the third appeared to be a mixture of the first and second rather than an intermediate stage.

Thus two variants of both Littledale and Whitehouse, namely, halo-producers and non-halo-producers, have been detected by their growth on immune-serum agar.

THE PROPERTIES OF THE VARIANTS.

The following further investigations were made on two selected colonies of Whitehouse, W. S/1 (halo-producer) and W. S/17 (non-halo-producer), in order to determine the general properties of the variants.

Staining Reactions.

The organisms were always Gram-negative.

Smears of 24-hour-old egg cultures of the halo-producer W. S/1, stained by Gram's method showed considerable variation in size amongst the individual cocci, with little tendency to diplococcal forms. Staining was irregular, some cocci being very deeply stained and others very faintly. As a general rule the larger cocci were the most deeply stained, although some of these took up hardly any stain at all.

Smears of the non-halo-producer, W. S/17, stained by Gram's method, showed much the same appearance as those of W. S/1, except that the cocci were definitely larger and stained more uniformly deep. In smears taken from this organism after passage through guinea-pigs, the cocci were all very large and of a uniform size and deep colour. This change was not so marked in W. S/1 after animal passage.

Capsule staining.—No method of staining has been successful in demonstrating capsules in either organism.

Growth in Liquid Media.

Broth.—In broth W. S/1 forms a very thin pellicle on the surface, which is easily shaken to the bottom of the flask. There is, at the same time, a general turbidity of the medium and a thin growth at the bottom of the flask.

W. S/17 grows without any pellicle and with a more even turbidity in broth, with a fine granular deposit at the bottom of the flask.

Peptone broth plus 5 per cent. normal rabbit-serum.—Growth in this medium is a test employed by Enders (1931) for the detection of " rough " and " smooth " meningococci. The results obtained with W. S/1 and W. S/17 in this medium

correspond exactly to those obtained by Enders with " S " and " R " organisms respectively.

W. S/1 grows in a pellicle " which ultimately disintegrates and falls to the bottom of the tube in the form of large flakes not easily disintegrated on shaking ".

The non-halo-producer W. S/17 grows in this medium exactly as described by Enders for his " R " variant. " In contrast to the ' S ', the ' R ' variant grows diffusely, or in extremely fine granules. A delicate granular deposit is laid down which is readily dispersed on shaking."

Growth on Solid Media.

Stab cultures and single colony growths of W. S/1 and W. S/17 were examined on the following media: Homologous immune horse-serum agar; Lange blood medium (Lange, 1929); human blood hormone agar (Huntoon, 1918); plain agar; 1 per cent. glucose agar; trypagar.

After incubation at 37° C. for 24 hours, differences were noted between the growths of these two organisms in all the above media except the plain and glucose agar, the greatest differences occurring on the immune-serum agar and the human blood hormone agar.

Growth on homologous immune-serum agar.—Both W. S/1 and W. S/17 grew well on this medium in 24 hours.

The surface of the growth of W. S/1 was slightly irregular, but very shiny and moist-looking. The edges of the colonies were even, and swept in wide curves. The growth shelved down gently at the edges, and by transmitted light a wide translucent fringe of growth was visible round the colonies, the centre being opaque. Haloes were visible round all colonies.

The non-halo-producer W. S/17 produced colonies with dull surfaces from which light was reflected unevenly. The surfaces of the colonies were pitted with numerous minute depressions and the growth sloped abruptly down to the medium. The colonies were irregular in outline and often exhibited bosses. By transmitted light the whole mass of the colonies was opaque, except for a very thin translucent edging. No haloes were developed by this organism in this medium.

Growth on human blood hormone agar.—The halo-producing organism W. S/1 grew very well and rapidly on this medium, with the production of many single colonies. The colonies were small, discrete and round, with smooth, regular edges. The surfaces were smooth and reflected light brilliantly. Towards the edges the surface of the colonies was usually heaped up and the centres were either flat or raised up. Completely surrounding the edges of the colonies there was a narrow rim of growth, which sloped gently up to the main mass.

W. S/17 grew more slowly and less luxuriantly on this medium and fewer single colonies developed. The colonies in general were larger than those of W. S/1 and the edges were irregular, with a general tendency to a round shape. In many cases the edges were scalloped and fissures ran back into the main mass of the growth. Under a lens the edges had the appearance of a relief map of a hilly coast. The surface was dull and unevenly wavy, and pitted by very fine depressions. The colonies grew as one mass of uniform thickness

and showed none of the characteristic features of W. S/1. Each colony differed from the others in outline and shape. The growth fell off sharply at the edges, and there was no rim of thin growth such as that seen round colonies of W. S/1.

The characteristics of the growths of W. S/1 and W. S/17 on hormone blood-agar, as described above, are similar to those described by Enders (1931) for " smooth " and " rough " meningococci respectively.

Other non-halo-producing colonies of both Whitehouse and Littledale produced growths on hormone blood-agar similar to that of W. S/17.

Agglutination and Absorption Tests.

Simple agglutination of suspensions of 98 single colonies of Littledale and Whitehouse, the details of which are recorded in a separate paper, failed to show any clear line of demarcation between the colonies, such as is seen in the Salmonellas (Andrewes, 1922). There was little difference between the reactions of suspensions of W. S/1 and W. S/17, both overlapping broadly into types I, II and IV.

Absorption of sera prepared from Whitehouse (both after weekly subculture on Dorset's egg medium and after 10 weeks' continuous growth in broth) with suspensions of W. S/1 and W. S/17 again failed to indicate any great differences between these organisms. Similarly, stock type sera were all absorbed to the same degree by suspensions of the two organisms.

Sera prepared from W. S/1 and W. S/17 both agglutinated stock type suspensions to the same degree, and absorption of these sera with both the homologous and heterologous suspensions completely removed all agglutinins.

The results of the agglutination and absorption tests on these two organisms are thus in contrast to the other evidence regarding them and have not confirmed the work of Enders, who claimed to have obtained an " S " serum which could not be absorbed by the " R " suspension, and an " R " serum which could be absorbed by both " S " and " R " suspensions.

It is possible that " rough " colonies from various sources differ considerably in the quantity of " S " antigen they contain, and *vice versâ*.

Precipitin Reactions.

Although the results of agglutinating suspensions of W. S/1 and W. S/17 are not what might be expected if we were dealing with " rough " and " smooth" variants, the results of precipitation reactions are definitely suggestive of this kind of variation.

Petrie's observation that the purified polysaccharide extract of the Shiga dysentery bacillus could produce haloes on homologous serum agar led him to the view that the rings were due to a precipitin reaction in the medium. When it was discovered that variants of meningococci existed which failed to develop haloes in homologous immune-serum agar, it was suggested that this might be due to the failure of these variants to produce a specific carbohydrate.

This problem was investigated first by attempting to repeat the halo tests as precipitin reactions, and secondly by attempting to isolate the specific body

from both the halo-producing organism (W. S/1) and the non-halo-producer (W. S/17).

These investigations are still incomplete, but as they throw light upon the nature of these variants, the results so far obtained are included here and will be treated separately.

Precipitin Tests on Antigens made from the Variants W. S/1 and W. S/17.

Antigens.—The organism was grown for 24 hours on fresh egg slopes, and then emulsified in about 2 c.c. distilled water for each slope. The watery suspensions were left at 37° C. for a week and then centrifuged for 2 hours at high speed. After dilution with an equal volume of 0·87 per cent. saline, they were incubated at 37° C. for a further 24 hours, and then re-centrifuged. Dilution with saline was necessary, as the first addition of saline produced a faint cloudiness which could be removed by centrifuging. Further dilution with saline produced no more clouding. The clear fluid left after the last centrifuging was used as antigen.

The reactions of the antigens made in the above manner from the halo-producer (W. S/1) and the non-halo-producer (W. S/17) respectively in the serum used in making the homologous immune-serum agar are shown in Tables I and II.

TABLE I.—*Precipitin Reaction in Homologous Immune Serum.* (1) *With W. S/1 Watery Antigen.*

		Dilution of antigen.						
		1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	Control.
Final dilution of serum	1/3	++	++	+	+	+	+	0
	1/6	++	++	+	+	+	tr+	..
	1/12	+	+	+	+	+	tr+	..
	1/24	+	+	+	+	+	tr	..

TABLE II.—*Precipitin Reaction in Homologous Immune Serum.* (2) *With W. S/17 Watery Antigen.*

		Dilution of antigen.						
		1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	Control.
Final dilution of serum	1/3	++	++	tr	tr	c	c	0
	1/6	++	tr+	c	c	0	0	..
	1/12	tr	c	c	0	0	0	..
	1/24	c	c	0	0	0	0	..

Note.—" c " in the above table indicates that the contents of the tube were cloudy, but no precipitate was present.

Tables I and II show that there was a certain amount of precipitation common to the two antigens, and that the antigen derived from W. S/1 (the halo-producer) precipitated with the serum at much higher dilutions than did the antigen obtained from the non-halo-producer, W. S/17.

Another series of tests was then performed to discover the end-point of the

reactions of the two antigens. It was found that at a serum dilution of 1/2 the reaction was positive with a dilution of the W. S/1 antigen of 1/1000, whereas in this dilution of serum no precipitation occurred with the W. S/17 antigen, even in a dilution of 1/10.

Extraction of the Specific Substance from W. S/1.

An attempt was made to extract the specific substance concerned in the above precipitin reactions from both the organisms W. S/1 and W. S/17.

Extraction was attempted mainly in the non-protein fraction of the growth. The method used was similar to that described by Zozaya (1932), with the exception that the reagents were not boiled.

The organism to be extracted was grown on egg for 24 hours and then emulsified in 2 per cent. sodium hydroxide. The protein was precipitated by acid and five volumes of absolute alcohol were added to the filtrate. A small precipitate settled out and was taken up in about 10 c.c. distilled water.

This final solution was used as antigen, and set up in a precipitin test against the homologous immune serum.

In a serum dilution of 1/6, the extract obtained from W. S/1 produced a precipitate at a dilution of 1/1,000,000.

Extraction of growths of W. S/17 have so far failed to produce any such active substance.

Discussion of Precipitin Results.

The simple test of comparing watery extracts of W. S/1 and W. S/17 shows that while there is some degree of precipitation common to both, the former possesses some reactive substance not possessed by the latter.

These results can be correlated with the formation and non-formation of haloes round these organisms in the following manner:

At a serum dilution of 1/20 in the serum-agar medium, W. S/1 develops haloes and W. S/17 does not. Referring to Tables I and II it will be seen that in a serum dilution of 1/24 the watery extract of W. S/1 gives a precipitate, whereas that obtained from W. S/17 does not produce anything beyond a faint cloud.

Thus, at a dilution of serum corresponding to that in the serum-agar medium, the results of the precipitin reactions parallel those of the halo tests on the serum-agar medium. It is therefore probable that the specific substance diffusing from growths of W. S/1 and producing haloes in the medium, is the same substance which is present in the watery extracts of W. S/1 and not in those made from W. S/17.

The reactions of the extract obtained from W. S/1 by the method of Zozaya indicate that this specific substance is of carbohydrate nature, as it was extracted from the non-protein fraction of the organism and did not give a positive Biuret reaction.

Injection of Animals with Variants.

Intraperitoneal injection of living organisms in large numbers, *e. g.* suspensions of the growth from two egg slopes, into guinea-pigs of about 250 gm. has so far produced death within 36 hours in 11 out of 12 animals inoculated

with the halo-producing organisms, and in 5 out of 14 inoculated with non-halo-producers.

This method of dosage is unsatisfactory owing to the difficulty of accurately measuring the dose, but so far inoculation with halo-producing organisms seems more fatal to guinea-pigs than inoculation with non-halo-producers.

The cause of death after intraperitoneal injection is uncertain. In only one case has the organism been recovered from the blood, although in some cases the blood was examined at half-hourly intervals.

After death from inoculation with the halo-producing colonies, the organism was in most cases recovered from the peritoneum, but after inoculation with non-halo-producers the organism was recovered from this source in only one case.

Meningococci were recovered from the base of the brain in 9 cases. Four of these had been inoculated with W. S/17 and 5 with W. S/1. One of the latter was killed 2 weeks after injection.

The results of post-mortem examinations of animals dying after inoculation of both variants indicated death from a rapid toxæmia. All the abdominal viscera were greatly injected, especially the suprarenals, which in many cases were black with hæmorrhages.

The brains were congested, but in no case showed any sign of active inflammation. The meninges were always normal in appearance, and there was no pus. Sections of brains revealed great congestion of the small vessels and some punctate hæmorrhages into the substance of the brain.

These experiments on the relative toxicity and virulence of the two variants are being continued.

Note on the Fermentation Reactions of Single Colonies of Whitehouse and Littledale.

A number of single colonies of both Littledale and Whitehouse were examined in broth containing the following sugars : glucose, maltose, saccharose, lactose.

The reactions were the same in all cases, glucose and maltose both being fermented within 24 hours and the other sugars remaining unaffected.

The reactions of the colonies W. S/1 and W. S/17 differed in that W. S/17 fermented glucose within 8 hours and the reaction was very much stronger than that produced by W. S/1, which did not ferment glucose until after 20 hours' incubation.

The tests were repeated several times with the same results.

The reactions of W. S/17 are in accordance with those described by Enders (1931) for the "rough" variant, *i. e.* the fermentation of W. S/17 is more acid and develops more rapidly than that produced by W. S/1.

Note on Various Sera and Freshly-isolated Strains.

Variants have been successfully isolated on agar media containing sera kindly supplied by the Wellcome Physiological Research Laboratories, and prepared by injecting horses with a number of freshly-isolated English strains supplied by the Ministry of Health, mostly type I, but including type II and some "indefinite" strains.

Meningococci of types I and III all develop characteristic haloes when grown on media containing the Wellcome sera, but organisms of type II do not.

On the Wellcome serum agar medium the "R" and "S" variants can be readily distinguished. The "S" organism grows as a smooth shiny mass with a faint yellowish, raised centre and a wide, flat edge. The "R" variant grows as an irregular mass with a dull surface, which has a wavy appearance. Freshly isolated type I and III organisms grow with definite "S" characteristics, but stock cultures of these types grow in colonies with more irregular edges than the "S" form, and with a shiny surface which is slightly folded and pitted. The appearance of stock cultures is probably due to their being a mixture of both variants (as in the case of Whitehouse).

Serum-agar plates made with human sera obtained from both acute and convalescent cases of cerebro-spinal meningitis act as good media for growing meningococci, but no haloes develop round any cultures.

So far rabbit-serum has not been satisfactory for making serum-agar media, but long immunization is probably necessary for the production of precipitin sera.

SUMMARY.

Certain variants of stock strains of meningococci can be detected by the characteristics of their growth on homologous immune-serum agar.

One variant develops haloes in this medium and the other does not.

Investigation of the properties of these variants has indicated that they are of "smooth" and "rough" nature respectively. This is supported by differences in growth in various media, in their fermentation and precipitin reactions and in their effects on guinea-pigs.

Agglutination and absorption reactions with sera prepared from the variants do not agree with the results obtained by Enders with his "S" and "R" forms. A tentative explanation of this discrepancy is offered.

These variants are being further investigated.

I wish to record my grateful thanks to Dr. A. D. Gardner for his criticism and kind help during the progress of this work, and also to Dr. G. F. Petrie, of the Lister Institute, Elstree, and Dr. R. A. O'Brien, of the Wellcome Physiological Research Laboratories, for kindly supplying the necessary sera, and to Dr. R. L. Vollum for his help in the biochemical portions of the work.

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EXPERIMENTAL OBSERVATIONS ON THE TOXIC EFFECTS OF STAPHYLOCOCCAL FILTRATES INTRODUCED ENTERALLY IN LABORATORY ANIMALS.

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DURING recent years cases of food-poisoning have been reported which, on investigation, have apparently been due to toxin formed by staphylococci (*aureus*, *albus* or *citreus* types) growing in the incriminated food material, *e. g.* chicken gravy, layered cream cake, cheese and milk. The symptoms described have been those of an acute gastro-enteritis developing a few hours after consumption of the contaminated food (Jordan, 1930; Jordan and Hall, 1931; Jordan, 1931).

Jordan and Hall (1931) were unable to produce symptoms of poisoning in laboratory animals by feeding with staphylococcal filtrates, but found that a severe and characteristic gastro-enteritis could be set up in human volunteers after consumption of 2 to 10 c.c. of culture-filtrates of staphylococci isolated from incriminated foods, and also from diverse other sources. Ramsay and Tracy (1931) fed kittens on milk-cultures of strains of *Staphylococcus aureus* known to have been responsible for gastro-enteritis in man. After several days these animals developed a diarrhœa which persisted until the milk culture was withdrawn from the food; in some cases blood and mucus appeared in the dejecta.

Woolpert and Dack (1933) produced symptoms of gastro-enteritis in *rhesus* monkeys by feeding with staphylococcal toxin obtained from known food-poisoning strains.

The object of the work recorded in this paper was to study this form of food-poisoning further by experimental methods.

METHODS.

Strains.

A strain of *Staphylococcus aureus* "Gon" (isolated from a carbuncle) was used in most of the experiments. In a few instances *Staphylococcus aureus* "Wood" was substituted. This latter strain was obtained from the National Collection of Type Cultures, and is the strain used by Burnet in his study of staphylococcal toxins (Burnet, 1929).

Preparation of Toxin.

The filtrates employed were prepared from cultures grown for 10 to 12 days in Walbum's (1922) liquid medium as recommended by Burnet (1930). The cultures were incubated in an atmosphere of 20–30 per cent. carbon dioxide.

In Vitro Tests of Filtrates.

Hæmolysin and leucocidin tests were used to estimate the activity of filtrates *in vitro*.

Hæmolysin Tests.

Serial dilutions of the filtrates ranging from 1 in 15 to 1 in 3840 were made up in 0·3 c.c. amounts in tubes, and 0·3 c.c. of a 1 per cent. suspension of washed rabbit red cells was added. The tubes were incubated at 37° C. for one hour; readings were then made, and also after the tubes had remained at room temperature overnight.

Leucocidin Tests.

The methylene-blue reduction test as originally employed by Neisser and Wechsburg (1901) was adopted. The white cells employed for the leucocidin tests were obtained from the leucocyte cream separated by centrifugalization from citrated ox blood (see Mackie, Finkelstein and Van Rooyen, 1932). By using a large amount of blood a preparation almost entirely free from red cells was obtained. 0·5 c.c. of a 5 per cent. leucocyte suspension was added to 0·25 c.c. of a series of dilutions of toxin in small test-tubes and also to two control tubes, one containing toxin heated at 60° C. for half an hour, and the other normal saline. The tubes were incubated at 37° C. for one hour; 0·05 c.c. of a 1 in 2000 dilution of methylene-blue was then added to each tube and the contents were transferred to agglutination tubes, and after the superimposition of paraffin seals they were reincubated for one hour, when readings were made.

ESTIMATION OF THE EFFECT OF GASTRIC CONTENTS ON TOXINS BY *IN VITRO* TESTS.

As it seemed possible that staphylococcal toxin, when introduced into the stomach, might be inactivated readily by the gastric secretion, especially by a secretion of acid reaction, the effect on the toxin of gastric contents of varying reactions was examined, the hæmolysin and leucocidin tests being used to estimate toxic activity. The technique already described for these tests was employed, the only modification being that an equal volume of gastric contents, or saline as a control, was added to the dilutions of filtrate and incubated for half an hour at 37° C.

It was observed that even slightly acid (pH 6·8) or slightly alkaline (pH 7·8) contents considerably impaired the activity of the filtrate, whereas a specimen with a reaction of pH 7·2 to pH 7·4 had no appreciable effect on the toxin. The toxic filtrate itself was pH 7·3. Typical results are given in the following table:

	End-titre of hæmolytic test.	End-titre of leucocidin test.
Toxin + equal volume of gastric contents with a reaction of pH 6·8	Only undiluted preparation active	Only undiluted preparation active.
Toxin + equal volume of gastric contents with a reaction of pH 7·8	Ditto	Ditto.
Toxin + equal volume of gastric contents with a reaction of pH 7·3	1 in 120	1 in 64.
Toxin + equal volume of normal saline	1 in 120	1 in 64.

ANIMAL EXPERIMENTS.

Introduction of Toxin into Stomach.

The animals used in the experimental work were rabbits and guinea-pigs. These animals were dieted for two days on green food and water, and on the day of the experiment they received no food till some hours after the introduction of the toxin.

In the preliminary work the required amount of toxin was introduced

directly into the stomach by means of a syringe attached to a No. 3 rubber catheter.

The majority of animals, apparently, were not affected by the toxin, but certain of them succumbed rapidly. Guinea-pigs were more easily affected than rabbits. Nine guinea-pigs and 5 rabbits were all given 8 to 10 c.c. of a toxic staphylococcal filtrate and symptoms of poisoning were produced in two guinea-pigs. One died within an hour after the inoculation of 8 c.c. of toxin. The second showed signs of illness and intense muscular weakness within an hour or two, and was killed after 6 hours and a post-mortem carried out. Both showed the pathological condition described later, though in the former it was more typical and pronounced than in the latter.

In subsequent work the catheter was introduced into the stomach as in previous experiments, but gastric juice was then aspirated by a syringe, and its reaction tested by means of a B.D.H. field capillator with phenol red as indicator. In general this preliminary sample was acid, and alkali (5 per cent. sodium bicarbonate) was introduced in order to neutralize the contents, a pH of 7·3 being aimed at. After a short interval a further specimen was aspirated and the pH tested. If still on the acid side more alkali was added; if, however, the reaction was now too alkaline, a small quantity of N/10 hydrochloric acid was added. This procedure was repeated until the samples showed a reaction of pH 7·3. Toxin was then introduced and the catheter withdrawn rapidly.

Seven guinea-pigs and one rabbit were all inoculated in this manner with 8 to 10 c.c. of toxin, and all died after intervals ranging from two minutes up to five days. General post-mortem findings in the different animals varied only in so far as the pathological condition was more pronounced in animals dying after several days than in those succumbing after a shorter interval. The prominent post-mortem features were intense congestion of the mucosa of the stomach and duodenum, associated with small hæmorrhages in the tissue and effusion of blood into the lumen, and congestion of various internal organs with hæmorrhages, *e. g.* kidney, liver and lung. The autopsy on one guinea-pig which died five days after inoculation revealed also hæmorrhage into the abdominal cavity.

Histological examination was made of the stomach-wall, liver and kidney, and in certain instances the lung, intestine and spleen. Sections of the stomach-wall showed intense congestion with areas of hæmorrhage, and in several cases extreme degeneration of glandular tissue. A similar condition was found in the intestinal wall, except that less hæmorrhage was present, and in addition to the preceding features severe catarrhal inflammation was observed. In sections of the liver, congestion, cloudy swelling and, in animals which survived several days, fatty degeneration were noted. The kidney showed intense congestion and some capillary hæmorrhages and, also, general cloudy swelling of the tubular cells. Sections of lung examined revealed marked congestion of the alveolar capillaries with slight hæmorrhages into the alveoli.

Two control experiments were performed on guinea-pigs. In one animal the gastric contents were made alkaline (pH 7·8), in the other acid (pH 6·8), previous to the addition of 8 c.c. of toxin. The health of the animals was apparently not impaired.

Intrarectal Inoculation of Toxin.

Guinea-pigs and rabbits were used for these experiments, and a No. 3 rubber catheter was passed a short distance into the rectum. In the first experiments the filtrate was introduced directly into the rectum, and the catheter withdrawn. The results of experiments performed by this method were uniformly negative.

In later experiments the rectum was irrigated with saline and, after a suitable interval, its reaction, usually slightly acid, was adjusted as nearly as possible to pH 7·3, by addition of 5 per cent. sodium bicarbonate and N/10 hydrochloric acid, in a manner similar to that already described for adjustment of the reaction of the stomach contents.

Two experiments were carried out by this method, and symptoms of poisoning resulted in both instances. One guinea-pig died two days after inoculation of 10 c.c. of toxic filtrate, the general post-mortem features being similar to those described in the preceding experiments, with the exception that the large intestine was deeply congested and contained mucus and blood. In addition, pronounced hæmorrhagic lesions were observed in the lungs. A second guinea-pig was inoculated with 10 c.c. of toxic filtrate and killed after two days. Intense congestion of the stomach, liver, spleen and kidneys was the prominent post-mortem feature along with hæmorrhages in the kidneys. The lungs in this case were apparently normal.

DISCUSSION.

The experiments recorded in this paper illustrate how laboratory animals such as rabbits and guinea-pigs may be protected normally against staphylococcal toxin when it is introduced directly into the alimentary tract. The results suggest that the inactivating effect of the acid contents of the stomach is an essential factor in this protection, and explain the failure of previous workers to reproduce experimentally in these animals the condition of "food-poisoning" by staphylococcal toxin as observed in the human subject. The sensitiveness of this toxin to slightly acid or alkaline reactions is of special interest. On the other hand, the local and general toxic effects produced by staphylococcal filtrates introduced into the stomach of guinea-pigs after neutralization of the contents, afford an interesting demonstration that the toxins of the staphylococci growing in certain articles of food may produce serious poisoning. It seems likely also that the hydrogen ion concentration of the stomach contents is a factor which determines the occurrence of such poisoning in the human subject, and this may explain the variable results observed by Jordan and Hall when volunteers were fed with milk containing staphylococcal toxin. The experiments indicate how small laboratory animals can, under certain conditions, be utilized for further inquiry into this form of food-poisoning, and may have a more general application in the study of the effects of other bacterial toxins on the alimentary tract.

SUMMARY AND CONCLUSIONS.

1. When staphylococcus toxin is added to gastric contents *in vitro* a slightly acid (pH 6·8) or a slightly alkaline (pH 7·8) reaction impairs its activity, whereas no inactivation occurs at pH 7·3.

2. Symptoms of poisoning can only occasionally be produced in guinea-pigs and rabbits when toxin is introduced directly into the stomach. Uniformly positive results are obtained in experiments in which the reaction of the stomach is adjusted to pH 7·3 at the time of introduction of the toxin. The animals die within five days, and post-mortem show signs of acute gastro-enteritis and marked congestion of internal organs, associated with hæmorrhage in the stomach and kidneys.

3. Symptoms of poisoning can be produced by intrarectal inoculation of toxin when the rectum had been irrigated with saline and the reaction adjusted to pH 7·3. Post-mortem features are similar to those obtained after introduction of toxin into the stomach. This method is not so effective as inoculation into the stomach.

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THE SIZE OF THE VIRUS OF FOWL PLAGUE ESTIMATED BY THE METHOD OF ULTRA-FILTRATION ANALYSIS.

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FOWL plague was first described as a virus disease by Centanni (1902), and has since been widely studied, particularly by continental workers. A summary of the relevant literature is given by Todd and Rice, M.R.C., 'System of Bacteriology', vol. vii, pp. 219–230. The causative principle or virus of this disease in the fowl is characterized by very high virulence,

even in extreme dilution, and also by the ready manner in which it passes through the commonly used types of bacteria-proof Berkefeld and Chamberland filter candles. It has for these reasons been generally regarded as being very small, although there is little evidence in the literature which enables a definite size value to be assigned to this virus. Andriewsky (1914) concluded that its size must be less than 2·3–2·5 mμ, this being Zsigmondy's figure for the size of the hæmoglobin molecule, since when filtering mixtures of hæmoglobin and infected fowl serum diluted in physiological saline, he obtained filtrates from 3 per cent. acetic acid collodion membranes which contained virus, although he could not detect the presence of hæmoglobin. However, no evidence is given that the several factors now known to determine filtration through membranes (Elford, 1933) were in any way appreciated. Furthermore, the test for this virus is much more delicate than the physical or chemical test for hæmoglobin, and in view of the wide difference between the maximum and average pore size in acetic acid collodion membranes (M.P.S. = 10 to 20 times the A.P.S.), it is possible for virus particles to pass through the largest pores in sufficient quantity to be detected, before enough hæmoglobin can pass for its presence in the filtrate to be demonstrated. These considerations cast a doubt upon the figure cited by Andriewsky being indicative of the size of the virus.

More recently Bechhold and Schlesinger (1931) made a 10 per cent. emulsion of infective fowl's brain in physiological saline, and after removing the coarsely suspended tissue fragments by preliminary centrifuging for 30 mins. at 2000 r.p.m., proceeded to subject the supernatant liquid to centrifugal sedimentation analysis. They calculated the size of the virus particles to be 120–130 mμ—a figure in marked contrast to that given by Andriewsky.

Further evidence upon the filterability and size of the virus of fowl plague is presented in this paper. Ultra-filtration analysis, employing carefully graded collodion membranes, has furnished data enabling the size of the elementary virus particles to be estimated.

Filtration technique.—The graded membranes and the filtration technique used have been essentially those employed in previous studies (Elford, 1931 ; Barnard and Elford, 1931 ; Galloway and Elford, 1931 ; Elford and Andrewes, 1932, *a* ; Elford, 1933). Reference should be made to these for the details. The membranes are graded in terms of their average pore size values (A.P.S.), which, as also the estimated particle sizes derived from them, are expressed in millionths of a millimeter (mμ). In previous papers the latter unit has been signified by μμ, which although in wide conventional use, does not conform strictly to the metric nomenclature. The correct symbol is mμ as adopted in this paper. Thus μ = micron = one thousandth of a milli-metre, and mμ = one thousandth of a micron = one millionth of a millimetre.

Strain of virus.—The strain of virus used in these studies was very kindly sent to us by Prof. Doerr, and was labelled " Stam Brescia ".

Test for virus.—In testing filtrates for the presence of virus, 1 c.c. of the liquid, unless otherwise stated, was injected intra-muscularly into a fowl. For this purpose three-months-old Plymouth Rock cockerels were used. If virus is present in the inoculum, the bird quickly succumbs to the disease, death taking place in two to three days. When a figure for the potency of

a filtrate was desired, tests for the virus were made upon serial tenfold dilutions in physiological saline.

Preparation of bacteria-free stock filtrates.—The virus is contained in high concentration by the body-fluids of the diseased fowl—pericardial fluid, serum, and blood-plasma. This obviated the necessity of making tissue emulsions, so that stock bacteria-free filtrates could be prepared by the direct filtration of the serum, suitably diluted in Hartley's broth, through a permeable membrane of A.P.S. 700–800 mμ. This effectively removed all fragments of cells, fibrin and contaminating bacteria, and furnished a filtrate of high potency in respect of the virus.

EXPERIMENTAL RESULTS.

The first experiments were made with the virus contained in the pericardial fluid of a fowl killed at the height of the disease. The potency of this fluid was such that 1/100,000 dilution proved infective. It was diluted with four times its own volume of Hartley's broth pH 7·6, and filtered through a 800 mμ membrane, yielding a stock filtrate which was infective in 1 : 10,000 dilution, but non-infective in 1 : 100,000 dilution. This filtrate served as starting material for further experiments with less permeable membranes. The results of these experiments are tabulated in Table I. They indicated that the end-point in membrane permeability for the virus lay between the limits 200 and 100 mμ.

TABLE I.—*Preliminary (Exploratory) Filtration Experiments.*

Membrane A.P.S. in mμ.	Membrane thickness (mm.).	Filtration pressure (cm. Hg.).	Volume filtered (c.c./sq. cm.).	Filtrate infectivity.
500	0·15	76	10	Undiluted, positive.
320	0·14	76	10	,, ,,
200	0·12	76	10	,, ,, / 1 : 10 negative.
The 500 mμ and 320 mμ filtrates were pooled and used for the following experiments. Infectivity of the mixed filtrates was such that 1 : 1000 was positive and 1 : 10,000 negative.				
120	0·16	76	10	Undiluted, negative.
65	0·12	76	7	,, ,,

The source of virus for the next experiments was infective blood-serum, which had been stored in the cold (3° C.) overnight, and become semi-gelatinous. After being diluted 1 : 4 with broth at pH 7·6, it was filtered through a sand and paper pulp filter in the hope that any fibrinous material would thereby be removed. The filtrate thus obtained was then filtered through a 800 mμ membrane at 20 cm. mercury pressure, to furnish a bacteria-free stock filtrate. Tests upon the infectivity of this filtrate showed that the 1 : 100,000 dilution was active, while 1 : 1,000,000 was inactive. This filtrate, after having been stored in the cold chest for three days, was filtered through a membrane of 150 mμ grade, using 76 cm. mercury pressure. 5 c.c. only of filtrate were

collected, but upon subsequent test this was found to be infective. The rate of filtration, however, had been very slow, suggesting that the storage of the stock filtrate in the cold had resulted in some further aggregation of the serum constituents. It was accordingly again diluted with an equal volume of broth and refiltered through an 800 mμ membrane prior to the following experiments summarized in Table II. These experiments demonstrated clearly that the virus could pass the 150 mμ membrane fairly readily, and that the permeability end-point was probably close to 125 mμ.

A further quantity of stock filtrate was prepared, on this occasion from infective serum and citrated blood-plasma from birds dying of the disease. The mixture of these pooled fluids was filtered through an 800 mμ membrane at 10 cm. mercury pressure. and yielded a filtrate which was slightly opalescent, and which proved to be infective in 1 : 100,000 dilution (the highest dilution tested). The pH of the filtrate was 8·2. After being kept for 5 days at 3° C., this filtrate was used in the following experiments given in Table III, its temperature being carefully adjusted to 20° C. before filtration. Again the rate of filtration was very slow, and the membranes soon became choked, suggesting, as before, considerable aggregation of the protein, probably of the fibrin of the plasma. Nevertheless the results were of definite importance, for, in addition to confirming the previous results in Table II, they also demonstrated that the serum proteins were able to pass the 125 mμ membrane in sufficient quantity to yield a precipitate with the sulphosalicylic acid test for protein, although the virus was wholly retained. This is direct evidence of the invalidity of Andriewsky's conclusion that the virus is probably smaller than serum protein.

TABLE II.

Source of virus.	Membrane A.P.S. in mμ.	Filtration pressure (cm. Hg.).	Volume filtrate (c.c./sq. cm.).	Membrane thickness (mm.).	Filtrate infectivity.
Blood-serum	150	76	10	0·15	2 c.c. undiluted, positive.
					1 c.c. ,, ,,
					1 c.c. 1 : 10 ,,
					1 c.c. 1 : 100 negative.
	125	76	10	0·16	2 c.c. undiluted, ,,
	65	76	7	0·12	2 c.c. ,, ,,

TABLE III.

Source of virus.	Membrane A.P.S. in mμ.	Membrane thickness (mm.).	Filtration pressure (cm. Hg.).	Volume filtered (c.c./ sq. cm.).	Filtrate test.
Pooled serum and citrated blood-plasma	150	0·14	76	2	Virus: 1 c.c. undiluted, positive. Protein: +++.
	125	0·16	76	1·5	Virus: 1 c.c. undiluted, negative. Protein: ++.

The presence of serum protein in the filtrates was determined by the sulphosalicylic acid test.

TABLE IV.

Source of virus.	Membrane A.P.S. in mμ.	Membrane thickness (mm.).	Filtration pressure (cm. Hg.).	Volume filtered (c.c./sq. cm.).	Infectivity of filtrate.	Protein in filtrate.
Fowl serum + blood-plasma	320	0·14	76	10	1 : 1,000,000 negative	+++
					1 : 100,000 positive	
					1 : 10,000 ,,	
					1 : 1000 ,,	
					Virus present in undiminished amount	
	150	0·14	76	10	1 : 100* positive	+++
					1 : 10 ,,	
	150	0·14	76	10	1 : 100,000 negative	+++
					1 : 10,000 ,,	
					1 : 1000 positive	
					1 : 100 ,,	
					1 : 10 ,,	
	125	0·16	76	2·5	Undiluted negative	++
				2·5	,, ,,	

* Highest dilution tested.

For the experiments of Table IV, designed to determine precisely the true end-point in membrane permeability for the virus, the serum-plasma filtrate was diluted 1 : 4 in broth, pH 7·6, to serve as the starting material. Its potency was such that 1 : 100,000 dilution was infective and 1 : 1,000,000 was non-infective. The results show that the virus can pass a 320 mμ membrane readily, the filtrate possessing undiminished potency; that while a 150 mμ membrane yields an active filtrate, there is a considerable proportion of virus retained by this membrane; and the 125 mμ membrane would appear to be very close to the limiting permeability, since it completely retains all the virus. The size of the virus would seem to be very much the same as that of *Staph. K* bacteriophage, which is just completely retained by a 110 mμ membrane, and has an estimated size 50–75 mμ (Elford and Andrewes, 1932, *b*). Some experiments were made using this phage as a control by adding it to the stock virus filtrate. Table V contains the results of experiments in which mixtures of fowl plague virus and *Staph. K* phage were filtered through membranes of grades 150 mμ and 125 mμ. The phage was estimated by the plaque count method on 1 per cent. agar plates, and the concentration has been expressed as the number of particles per c.c. It is evident that the virus of fowl plague is probably very slightly larger than *Staph. K* phage, but there is very little difference. The results with the mixture fit in excellently with the respective individual end-points—*Staph. K* phage retained by a 110 mμ membrane and the virus of fowl plague by a 120 mμ membrane.

Taking 120 mμ to be the end-point in membrane grade for fowl plague, the probable size of the virus, on the basis of calculation adopted for these membranes (Elford, 1933) may be given as 60–90 mμ, *i. e.* 0·5–0·75p, where p is the limiting pore size.

DISCUSSION.

The outstanding feature of the present series of filtration experiments with fowl plague has been the clearly defined end-point in membrane permeability.

TABLE V.—*Filtration of Mixtures of Fowl Plague Virus and* Staph. K *Phage.*

System filtered.	Membrane A.P.S. in mμ.	Membrane thickness (mm.).	Filtration pressure (cm. Hg.).	Test on filtrate.
Infective serum and blood-plasma filtrate diluted 1 : 4 in broth was filtered through a 320 mμ membrane, yielding a filtrate infective in 1 : 100,000 dilution. This latter filtrate was mixed with its own vol. of *Staph. K* phage filtrate. The phage in the mixture was 5×10^7/c.c.	150	0·14	152	Virus : 1 : 100 + 1 : 1000 + 1 : 10,000 — Phage : + ; 6×10^6/c.c.
	125	0·16	152	Virus : Undiluted + 1 : 10 — Phage : + ; 7×10^3/ c.c.
	125	0·16	152	Virus : Undiluted + 1 : 10 — Phage : + ; 5×10^3/c.c.
	125	0·16	76	Virus : Undiluted — Phage : + ; 3×10^3/c.c.
Control. Virus only. The 320 mμ membrane filtrate diluted with its own vol. broth	125	0·16	152	Virus : Undiluted — 1 : 10 —
	125	0·16	76	Virus : Undiluted — 1 : 10 —
Control. Phage only. Phage filtrate diluted with own vol. broth giving 5×10^7/c.c.	125	0·16	76	Phage : + ; 5×10^3/c.c.

In each experiment 10 c.c. were filtered per sq. cm. membrane.

Thus the virus passed through a 320 mμ membrane in undiminished concentration, but with a 150 mμ membrane, although the filtrate was still strongly active, the percentage drop in the degree of infectivity was fully 90 per cent. In all the experiments in which a straight virus suspension was filtered through 125 mμ membranes, the filtrates were consistently inactive with filtration pressure up to two atmospheres. However, in two experiments in which *Staph. K* phage was mixed with the virus, a trace of the latter was able to pass through a 125 mμ membrane. The evidence showed that *Staph. K* phage filtered somewhat more readily than the virus, and at two atmospheres pressure actually assisted a few virus particles to get through the 125 mμ membrane. It seems possible that the virus particle, being only very slightly larger than the phage particle, and just retained by this grade of membrane when in free suspension, is able, when following in the wake of a phage particle which can just get through the pore, to traverse the pore itself, provided always that the kinetic velocity of the filtering stream is sufficiently high, *i. e.* under favourable conditions of filtration pressure.

The size of the virus estimated here differs greatly from that given by Andriewsky, and our experiments have conclusively shown that the serum proteins are very much smaller than the virus. This, of course, follows directly from the respective end-points in membrane permeability, viz. 120 mμ for fowl plague virus and 9 mμ for serum proteins. The suggestion of Mrowka (1914) that the virus of fowl plague is a virulent globulin—a conclusion to which he was led by the fact that he centrifuged down the infectivity along with globulin precipitates—is also discounted.

Our value for the size of the virus is, on the other hand, definitely less than that given by Bechhold and Schlesinger (1931) derived from centrifugal analysis data. It may be recalled that the size of vaccinia virus given by these authors in the same paper, 210–230 mμ, was definitely greater than the size of the virus estimated by Elford and Andrewes (1932, *a*) from ultra-filtration, 125–175 mμ. The latter value has received some confirmation in the work of Barnard, who by ultra-violet photography arrives at the value 160–180 mμ for the maximum size of vaccinia virus. The results would suggest that the estimate of Bechhold and Schlesinger for the size of these two viruses is slightly high in both cases.

SUMMARY.

The size of the elementary virus particles of fowl plague, as they exist in the body fluids of the fowl, has been estimated by ultra-filtration analysis, using graded collodion membranes, to be 60–90 mμ. Control experiments in which *Staph. K* bacteriophage (size 50–75 mμ) was admixed with the virus suspension confirmed the close relationship in size between this phage and the virus. Differentiation of their filterabilities was only just possible.

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THE GONADOTROPIC HORMONES (ρ-FACTORS). V. THE EFFECT OF LARGE DOSES ON SUBSEQUENT FERTILITY.

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The therapeutic use of gonadotropic hormones has been discussed in a recent communication by Johnstone, Wiesner and Marshall (1932). Only a few preliminary experiments were described there, which gave some indication of the line of attack on some clinical problems which was likely to be the most fruitful; this line is now being followed up.

The earlier extracts used for this work were prepared by the use of sulphosalicylic acid from human full-term placenta as described in Part I of this series (Wiesner and Marshall, 1931), and were of extremely variable potency. For nearly two years extracts for clinical purposes have been prepared from pregnancy urine by the method described in Part IV of the series (Marshall, 1933).

One of the criticisms levelled against a clinical investigation of this nature has been that it is a very dangerous procedure to adopt in the light of our present knowledge of possible pathological changes in the ovary which may even be permanent. The work to be described here affords strong evidence that the breeding capacity of mice is not impaired by the injection of very large doses of ρ-factors (many times the doses which have been given to human beings in the treatment of dysmenorrhœa, etc., within a similar period), and also suggests that the percentage of "runts" in an unselected stock may actually be decreased. The accurate determination of the degree to which this can be effected under the conditions to be described would require a very large series of experimental animals (both controls and injected), and no claim is made to have established this point in the present work.

EXPERIMENTAL.

Care and feeding of animals.—In any investigation of fertility constancy of diet is obviously an important factor, just as it is in the investigation of the growth-promoting hormone of the anterior lobe of the pituitary. In the present series flaked maize was given to all animals as the staple food, supplemented by skim milk given three times a week, and brown bread once a fortnight, since maize alone is not a complete food; water was given when milk was not provided. The above diet was found to be ample to maintain the animals in good condition, and with two exceptions, which will be referred to later, they all weighed 25–30 gm. Nevertheless the diet is not a rich one, and it is hardly conceivable that it could have influenced fertility on this account.

Extracts.—The same phosphate-free ρ-factor preparation (25 mμ per mg.) was used throughout the experiment, and was made up in 0·85 per cent. saline, and adjusted to pH 7·0 (1 c.c. contained 100 mμ). The approximate standardization was carried out as described in a previous communication (Marshall, 1932).

Effect on immature mice.—Fifteen mice (aged 22 days) were taken in this preliminary experiment, 5 of which were controls. The remaining 10 received 9 injections of 0·4 c.c. (40 units) in the course of 10 days. During the course of a fortnight from the beginning of the experiment 5 animals were killed at intervals; all showed great ovarian hypertrophy, many blood points and a large number of corpora atretica. The remaining 5 injected animals were left along with the 5 controls for 6 weeks; the two groups were then placed separately with two males. A large number of plugs were obtained without pregnancy ensuing (more frequently in non-injected animals)—not an unusual finding for the time of year (November–December), and one which accorded with the observations of other workers in the laboratory on normal non-injected mice. By January 11th four litters were obtained in each series; the details are given in the following table:

TABLE I.

	Controls.		Injected.	
No.	Date.	Litter size.	Date.	Litter size.
1	1.i.33	6	8.xii.32	8
2	3.i.33	4	17.xii.32	7
3	10.i.33	6	22.xii.32	5
4	11.i.33	11	3.i.33	9

All animals were placed with a male on November 15th, 1932. The length of pregnancy was normal in all the injected animals (19 days). It is to be noted that the injected animals tended to produce litters at an earlier date than the controls. Litter size is very variable even in normal animals.

Effect on mature mice.—For the purpose of this experiment 20 mature females (virgins about 14 weeks) were ordered from a breeder and used without any attempt being made to examine their œstrus cycle. In addition, 4 animals from the previous experiment were taken from the young at 3–4 weeks, and included in the present one. All the animals received 9 injections in the course of 10 days, with a total of 360 mμ. Four days after the last injection a potent male was placed with every 5 females. From the 20 unselected mice 13 had litters within 5 weeks, 15 within 8 weeks, and 18 within 10 weeks. Of the other group of 4 animals, only one had a litter within 4 weeks, and the remaining 3 within 8 weeks; the size of the litters was 5, 8, 8 and 11. Of the 18 unselected mice which became pregnant, one had a litter of 10, two had litters of 9, 6 had litters of 8, and the remaining 5 had litters of 5 or less; the average litter size was 6·9—a figure which should probably be somewhat higher, since one or two of the young were certainly eaten before being counted.

DISCUSSION.

In the series of experiments described above there are one or two rather striking observations. Even with the somewhat limited number of animals used, it would seem to be definitely established that the administration of very large doses of ρ-factors in mice over a period of 10 days, which corresponds to the period of a course of injections in women being treated for dysmenorrhœa, has no permanent sterilizing effect, since 90 per cent. of the unselected stock used (18 out of 20) had litters within 10 weeks. Although it is extremely difficult to give a definite value to the percentage of litters obtainable with a normal non-injected stock, it is highly improbable that it would be higher than 90 per cent. It is further to be noted that the remaining two of the series, which did not have litters within the period stated, were definitely puny, under-developed animals of not more than 20 gm., from some cause which could not be attributed to the hormone solution injected. For these reasons it would appear possible that the administration of ρ-factors under the conditions described above, either while the animals are still immature or in adult mice, may even increase the fertility of an unselected stock. However, further work will be required to establish this point definitely.

It was recently reported by Mandelstamm and Tschaikowsky (1931) that

they were able to inhibit ovulation for 5–6 cycles by a single injection of 10 units of Prolan; in a series of 20 mice they state that they obtained no pregnancies in the course of 28 days by placing the animals with a male 4 days after the injection, whilst under similar conditions 29 out of 36 non-injected females became pregnant. The specimen of hormone used by these workers was stated by them to contain more Prolan B than A, but no indication is given of the method of standardization.

It will be seen that in the work described here 13 out of 20 unselected animals must have become pregnant within 15–16 days, since that number of litters was obtained within 34 days. The author is of the opinion that the proportions of ρ_1 and ρ_2 vary in different hormone preparations obtained from different batches of urine—a point which is to be further investigated—but all standardizations have been referred to in terms of "luteinizing" units, and there is no doubt that the specimen used was rich in ρ_2 (Prolan B).

No attempt has been made to determine the sex ratio, since this was of secondary importance from the point of view of the present therapeutic use. Nevertheless the observations of Kraft (1932) on the effect of pituitary transplants in young rats are of great interest, and show a greater effect on litter size than is recorded in the present communication. This increase was found to be more pronounced in the second generation, and the sex ratio was very significantly reduced. The exact amount of hormone given was not determined (although from the clinical aspect this is important), nor was any indication given of the percentage of litters obtained, but the results favour the conclusions drawn by the author from the present investigation that the therapeutic use of ρ-factors is not only safe, but eminently desirable. The dose of hormone which has been given in any clinical trial has been less, even in absolute amount, than that administered to mice in the present experiments, whilst, taking into account body-weight, it is merely an infinitesimal proportion.

In one instance an immature monkey, about 18 months old, was injected with very large doses of ρ-factors; 300–500 mμ were administered daily intramuscularly for 2–3 weeks. After a gap of 3 weeks without injections, further quantities of from 600–1200 mμ were given on alternate days for a further 2–3 weeks. At operation 3 days after the last injection it was seen that no hyperæmia or luteinization of the ovary had been produced; only a few enlarged follicles were present.

SUMMARY.

(1) The effect of the injection of very large doses (360 mμ) of ρ-factors on the subsequent fertility of both immature and adult female mice has been investigated.

(2) In a group of 20 unselected mice (mature females) injected with ρ-factors, 65 per cent. of litters were obtained within 5 weeks (pregnancy therefore occurring within 16 days), and 90 per cent. within 10 weeks of placing with males. This does not accord with the findings of Mandelstamm and Tschaikowsky, who used a Prolan preparation, and do not mention standardization methods. The percentage of litters from all 24 animals in the experiment was 91·7 per cent. within 10 weeks.

(3) No luteinization or hyperæmia of the ovaries occurred in an immature monkey injected with more than 10,000 mμ of hormone.

(4) The evidence obtained in the present investigation strongly supports the view that gonadotropic hormone therapy is not only safe, but a natural and important development of laboratory experimentation.

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THE INFECTIVE AGENT IN TUMOUR FILTRATES: A FURTHER INVESTIGATION BY MEANS OF ANTISERA TO NORMAL TISSUES.

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From the Medical Research Council's Field Laboratories, Mill Hill, London.

Received for publication May 30th, 1933.

ELSEWHERE (Gye and Purdy, 1931) we have described experiments in which we used anti-fowl immune-serum made by injecting goats with cell-free extracts of fowl embryos. The experiments showed that, in the presence of fresh guinea-pig serum, immune sera prepared from normal fowl tissues would neutralize extracts of each of the few fowl tumours available, including extracts of the Rous sarcoma No. 1. The experiments showed, too, that the effective antibody could be absorbed from these anti-fowl sera by minced fowl embryo, but not by minced duck embryo. Particular attention was given to the Fujinami myxosarcoma, a tumour which grows in both ducks and fowls, and it was found that anti-fowl serum would neutralize extracts of fowl-grown but not of duck-grown Fujinami tumours. From the various experiments it was concluded that an essential component of the infective agent present in a tumour filtrate is something which has the same species-specificity as the bird that carried the tumour, and is antigenically identical with something which can be extracted from normal tissues. The first part of the present paper presents further evidence in support of this early conclusion. It deals with experiments which are complementary to those already described; an anti-duck serum has been prepared and used against duck-grown and fowl-grown Fujinami tumours. The second part of the present paper deals with experiments in which the species-specific component of a tumour agent has been investigated more closely than hitherto.

In all the experiments to be described in the present paper the same general method of testing filtrates by means of immune sera has been employed. Normal and immune sera have been allowed to act upon tumour filtrate, usually at incubator temperature. We have used 3 volumes of serum to 1 volume of filtrate, but no claim is made that these proportions are the best. We have not investigated systematically the effects of varying the serum-filtrate proportions over a wide field. We have been content to adopt proportions which we knew to be suitable. A more important, indeed the paramount consideration in all our experiments has been the provision of the most careful control inoculations. Rigorous control of experimental results is compulsory for two reasons: first because chickens differ in their resistance to the action of a given filtrate; and secondly, because it is only with the most potent of immune sera that tumour formation is absolutely stopped—usually there is no more than a partial inhibition. The plan of giving multiple inoculations in each chicken of an experiment satisfies the requirements and has been adopted throughout. Our method of testing immune sera is illustrated in the following experiment, which is selected as typical of those in which the action of anti-duck serum was investigated. The experiment was carried out on August 28th, 1931.

Four different sera were available. These were allowed to act upon two tumour filtrates in the absence as well as in the presence of fresh guinea-pig serum. One of the filtrates had been prepared from a Fujinami tumour grown in a fowl, and the other from a Fujinami tumour grown in a duck. The normal control serum had been obtained from Goat 23 before that animal had received any injections whatever, and the anti-duck serum had been obtained from this same animal after a series of injections of cell-free extracts of whole duck embryos had been given. The anti-fowl serum had been obtained from Goat 19 after that animal had received doses of cell-free extracts of whole fowl embryos. The anti-tumour immune serum had been obtained from Goat 17—an animal which had received a series of injections of cell-free extracts of fowl-grown Fujinami myxosarcoma. All 4 sera had been filtered through candles and then heated at 55° C. for 30 minutes. The interval between successive immunizing doses was usually 5 to 7 days. No difficulty has been found in the production of such immune sera, hence the omission of a more complete account of the immunization of Goats 17, 19 and 23. The general method was the same as that used in our earlier experiments, and already described in detail in the publication referred to above.

The mixtures in which the action of our anti-duck immune serum was tested and compared with the action of the other 3 sera were prepared as indicated in the following tables. The preliminary mixtures merely formed a convenient intermediate step. All quantities are given in cubic centimetres.

Preliminary mixtures.				
Z.	Y.	X.	W.	
5 c.c.	—	5 c.c.	—	Filtrate of duck-grown Fujinami tumour.
—	5 c.c.	—	5 c.c.	Filtrate of fowl-grown Fujinami tumour.
5 c.c.	5 c.c.	—	—	Complement (fresh guinea-pig serum).
—	—	5 c.c.	5 c.c.	Saline.

(3) No luteinization or hyperæmia of the ovaries occurred in an immature monkey injected with more than 10,000 mμ of hormone.

(4) The evidence obtained in the present investigation strongly supports the view that gonadotropic hormone therapy is not only safe, but a natural and important development of laboratory experimentation.

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5 c.c.	5 c.c.	—	—	Complement (fresh guinea-pig serum).
—	—	5 c.c.	5 c.c.	Saline.

Final mixtures.													
A.	B.	C.	D.	E.	F.	G.	H.	J.	K.	L.	M.	N.	P.	
2	2	2	2	—	—	—	—	—	—	—	—	—	—	Comp.+filt. of duck tum. (mixt. Z).
—	—	—	—	2	2	2	2	—	—	—	—	—	—	Comp.+filt. of fowl tum. (mixt. Y).
—	—	—	—	—	—	—	—	2	2	2	—	—	—	Saline+filt. of duck tum. (mixt. X).
—	—	—	—	—	—	—	—	—	—	—	2	2	2	Saline+filt. of fowl tum. (mixt. W).
3	—	—	—	3	—	—	—	3	—	—	3	—	—	Normal serum of Goat 23.
—	3	—	—	—	3	—	—	—	3	—	—	—	—	Anti-duck serum of Goat 23.
—	—	3	—	—	—	3	—	—	—	—	—	3	—	Anti-fowl serum of Goat 19.
—	—	—	3	—	—	—	3	—	—	3	—	—	3	Anti-tumour serum of Goat 17.

The final mixtures were incubated aërobically for 90 minutes at 37° C. and then tested for potency by means of bird inoculations. All inoculation doses were 1·0 c.c. The birds used, the sites injected and the results observed are all shown in the accompanying figures. It will be seen that Mixtures A, B, C and D were tested in Fowls 1, 2, 3 and 4; Mixtures E, F, G and H were tested in Fowls 5, 6, 7 and 8; Mixtures J, K and L were tested in Ducks 69, 70, 73 and 74; whilst Mixtures M, N and P were tested in Ducks 75, 76, 77 and 78. No fallacy is introduced by using fowls for some of the tests, and ducks—actually they were young ducklings—for the remainder. In experiments not here described fowls only or ducks only have been used throughout. In this kind of experiment the answer is the same whichever kind of bird—fowl or duck—is used.

It will be seen from Figs. 1 and 2, which summarize the illustrative experiment just described, that an immune serum prepared by injecting goats repeatedly with extracts of fowl-grown tumours neutralized both filtrates, even that prepared from the tumour grown in a duck. It will be seen, too, that neutralization was complete in the absence as well as in the presence of complement. This antigen, which is common to duck-grown and fowl-grown Fujinami tumours, must be extrinsic to both species of birds. In an earlier publication (Gye and Purdy, 1931) we have identified this antigen as the living tumour virus (or product of virus). It will be observed from the figures, too, that the filtrate of duck-grown tumour was neutralized—or nearly so—by the anti-duck serum in the presence of complement, though not in the absence of complement. Conversely, the filtrate of fowl-grown Fujinami tumour was neutralized by the anti-fowl serum in the presence of complement, and was totally unaffected by the anti-duck serum.

In other experiments it has been shown that minced duck-embryo tissue absorbs the neutralizing antibody from anti-duck serum, but not from anti-fowl serum; the reverse of this is true for minced fowl embryo. Neither minced duck embryo nor minced fowl embryo removes the antibody which neutralizes filtrates of both fowl and duck tumours, and which is present in the serum of goats that have been injected repeatedly with tumour extracts.

Thus the evidence is now completed for the view that when a Fujinami tumour is passed from a fowl to a duck the species-specific component of the infective agent changes, fowl specificity giving place to duck; and that when a Fujinami tumour is transferred from a duck to a fowl the process is reversed, the duck-specific component giving place to one having the same species specificity as

EACH INOCULUM CONTAINED A STANDARD AMOUNT OF FILTRATE OF **DUCK-GROWN** MYXOSARCOMA, A STANDARD AMOUNT OF COMPLEMENT, AND A STANDARD AMOUNT OF SERUM. THE NATURE OF THE SERUM USED IS SHOWN BELOW.	FOWL 1. DIED AT 21 DAYS	FOWL 2 DIED AT 22 DAYS	FOWL 3 DIED AT 24 DAYS	FOWL 4. DIED AT 23 DAYS
NORMAL SERUM TAKEN FROM GOAT 23 BEFORE THE ANIMAL HAD BEEN IMMUNIZED.	R. BREAST	R. BREAST	R. BREAST	R. BREAST
SERUM TAKEN FROM GOAT 23 AFTER THE ANIMAL HAD BEEN IMMUNIZED WITH EXTRACTS OF **DUCK** EMBRYO.	L. BREAST	L. BREAST	L. BREAST	L. BREAST
SERUM TAKEN FROM GOAT 19 AFTER THE ANIMAL HAD BEEN IMMUNIZED WITH EXTRACTS OF **CHICK** EMBRYO.	R. LEG	R. LEG	R. LEG	R. LEG
SERUM TAKEN FROM GOAT 17 AFTER THE ANIMAL HAD BEEN IMM. WITH EXTRACTS OF FOWL-GROWN **MYXOSARCOMA.**	L. LEG NIL	L. LEG NIL	L. LEG NIL	L. LEG NIL

EACH INOCULUM CONTAINED A STANDARD AMOUNT OF FILTRATE OF **FOWL-GROWN** MYXOSARCOMA, A STANDARD AMOUNT OF COMPLEMENT, AND A STANDARD AMOUNT OF SERUM. THE NATURE OF THE SERUM USED IS SHOWN BELOW.	FOWL 5. DIED AT 18 DAYS	FOWL 6. DIED AT 19 DAYS	FOWL 7. DIED AT 20 DAYS	FOWL 8 DIED AT 21 DAYS
NORMAL SERUM TAKEN FROM GOAT 23 BEFORE THE ANIMAL HAD BEEN IMMUNIZED.	R. BREAST	R. BREAST	R. BREAST	R. BREAST
SERUM TAKEN FROM GOAT 23 AFTER THE ANIMAL HAD BEEN IMMUNIZED WITH EXTRACTS OF **DUCK** EMBRYO	R. LEG	R. LEG	R. LEG	R. LEG
SERUM TAKEN FROM GOAT 19 AFTER THE ANIMAL HAD BEEN IMMUNIZED WITH EXTRACTS OF **CHICK** EMBRYO.	L. BREAST	L. BREAST	L. BREAST	L. BREAST
SERUM TAKEN FROM GOAT 17 AFTER THE ANIMAL HAD BEEN IMM. WITH EXTRACTS OF FOWL-GROWN **MYXOSARCOMA.**	L. LEG NIL	L. LEG NIL	L. LEG NIL	L. LEG NIL

FIG. 1.—In the presence of complement anti-duck serum neutralized extract of duck tumour, but not of fowl; anti-fowl serum neutralized extract of fowl tumour, but not of duck; anti-tumour serum neutralized both extract of duck tumour and extract of fowl tumour.

the new host—the fowl. Incidentally, too, the existence of the species component is now directly demonstrated in filtrate of a duck tumour; hitherto it has been actually demonstrated only in fowl tumours. The investigation

EACH INOCULUM CONTAINED A STANDARD AMOUNT OF FILTRATE OF **DUCK-GROWN** MYXOSARCOMA AND A STANDARD AMOUNT OF SERUM. THE NATURE OF THE SERUM IS SHOWN BELOW. COMPLEMENT WAS NOT PRESENT	DUCK 69 DIED AT 10 DAYS	DUCK 70 DIED AT 10 DAYS	DUCK 73 DIED AT 12 DAYS	DUCK 74 DIED AT 11 DAYS
NORMAL SERUM TAKEN FROM GOAT 23 BEFORE THE ANIMAL HAD BEEN IMMUNIZED	R. LEG	R. LEG	R. LEG	R. LEG
SERUM TAKEN FROM GOAT 23 AFTER THE ANIMAL HAD BEEN IMMUNIZED WITH EXTRACTS OF **DUCK** EMBRYO	L. LEG	L. LEG		
SERUM TAKEN FROM GOAT 17 AFTER THE ANIMAL HAD BEEN IMM. WITH EXTRACTS OF FOWL-GROWN **MYXOSARCOMA**			L. LEG NIL	L. LEG NIL

EACH INOCULUM CONTAINED A STANDARD AMOUNT OF FILTRATE OF **FOWL-GROWN** MYXOSARCOMA AND A STANDARD AMOUNT OF SERUM. THE NATURE OF THE SERUM IS SHOWN BELOW. COMPLEMENT WAS NOT PRESENT.	* DUCK 75 DIED AT 8 DAYS	DUCK 76 DIED AT 9 DAYS	DUCK 77 DIED AT 12 DAYS	DUCK 78 DIED AT 12 DAYS
NORMAL SERUM TAKEN FROM GOAT 23 BEFORE THE ANIMAL HAD BEEN IMMUNIZED	R. LEG	R. LEG	R. LEG	R. LEG
SERUM TAKEN FROM GOAT 19 AFTER THE ANIMAL HAD BEEN IMMUNIZED WITH EXTRACTS OF **CHICK** EMBRYO.	L. LEG	L. LEG		
SERUM TAKEN FROM GOAT 17 AFTER THE ANIMAL HAD BEEN IMM. WITH EXTRACTS OF FOWL-GROWN **MYXOSARCOMA**.			L. LEG NIL	L. LEG NIL

FIG. 2.—**In the absence of complement anti-duck serum failed to neutralize extract of duck tumour, and anti-fowl serum failed to neutralize extract of fowl tumour; anti-tumour serum, as before, neutralized both extracts.**

cannot be extended to other duck tumours, since none are available. The general significance attached to such observations as those illustrated in the experiment described above has already been discussed at length (Gye and Purdy, 1931), and demands no further comment.

The problem which now presents itself is the elucidation of the more intimate nature of this antigen which is present in normal embryos, and which gives rise to antibody capable of neutralizing the active agent in a tumour filtrate. What is the exact source of this antigen? We know that it has species specificity; has it also cell specificity? Is it derived from cells or from tissue fluids—or even from both? Is the antibody which neutralizes filtrate of a fowl sarcoma the same as that which neutralizes, for instance, filtrate of a fowl endothelioma, or have we been dealing hitherto with a multiplicity of antibodies? These are questions to which answers have been sought by the experimental method, as will now be described.

Erythrocytes are the body-cells most readily obtained in quantity and practically unmixed with other cells; blood-plasma is the body fluid most easily obtained. Both have the species specificity of the animal from which they come, and both readily give rise to their corresponding antibodies. Blood-plasma and extract of blood-cells were therefore selected for investigation. The first step was to determine whether either contains the sought-for antigen. This necessitated the preparation of immune sera.

Samples of serum were obtained from three normal goats for control purposes. Then each goat was immunized by means of repeated doses of its selected antigen. One goat received repeated doses of extract prepared from fowl red cells, another received doses of fowl blood-plasma, and the third received doses of duck blood-plasma. Inocula were always freshly prepared, and were always prepared in a standard way. Thus when plasma was required, 10 c.c. of blood was taken directly from the wing vein of bird—duck or fowl, as the occasion demanded—into a syringe, which, to prevent clotting, already contained 10 c.c. of a 1·5 per cent. solution of sodium citrate in saline. The syringe was shaken to mix its contents thoroughly, and then the contents were discharged into a flask containing 100 c.c. of saline. After this diluted blood had been spun, the supernatant plasma was taken off, filtered free of any remaining cells, and used for the injection. When extract of red cells was required, the cells spun down from blood which had been citrated and diluted as described were washed several times in saline in the usual way. The cells were washed to remove the last traces of plasma, but it became clear later that the experimental results would have been the same had they remained unwashed. The washed red cells were ground with sand in the way customary for tumour tissues and then were extracted with saline, 100 c.c. of saline being used for each 5 c.c. of sedimented red cells. Finally, the crude extract thus prepared was spun free of gross particles, filtered, and used for the injection. The following tables show the goats used and the doses given. The period of immunization extended over the later months of 1931 and the early months of 1932.

Each goat was bled at the end of its immunization periods to provide samples of its serum. All sera, both normal and immune, were filtered at the time of preparation, and were afterwards heated at 55° C. for 30 minutes.

The normal and immune sera of these three goats have all been tested for their power of neutralizing active agent in extracts of Fujinami's tumour. Details of each individual test need not be given, since the methods employed were always closely similar to those used in the experiment which has been summarized in Figs. 1 and 2. A single experiment will be described as

representative of all, but for convenience we give first a summary of the results yielded by this kind of experiment.

The sera of goats immunized with blood-plasma—the one with fowl-plasma and the other with duck—both failed to neutralize, and the failure was complete, no matter whether the filtrate had been made from duck-grown or from fowl-grown tumours, and no matter whether complement were present or not. The immune sera, though rich in precipitins, contained no detectable anti-tumour antibody. The experiments were repeated a number of times, but

Immunization of Goat 24 with Extract of Fowl Red Cells.

Sept. 23	Goat bled.	Nov. 6	Goat bled.
,, 24	25 c.c.	Feb. 2	160 c.c.
,, 29	50 ,,	,, 4	130 ,,
Oct. 5	50 ,,	,, 15	Goat bled.
,, 12	70 ,,	Mar. 9	110 c.c.
,, 20	40 ,,	,, 10	80 ,,
,, 27	45 ,,	,, 20	Goat bled.

Immunization of Goat 26 with Fowl Plasma.

Dec. 8	Goat bled.	Dec. 29	80 c.c.
,, 9	5 c.c.	Jan. 5	Goat bled.
,, 12	10 ,,	Mar. 9	110 c.c.
,, 17	20 ,,	,, 10	120 ,,
,, 23	40 ,,	,, 20	Goat bled.

Immunization of Goat 27 with Duck Plasma.

Dec. 7	Goat bled.	Dec. 28	80 c.c.
,, 7	5 c.c.	Jan. 5	Goat bled.
,, 11	10 ,,	Mar. 10	100 c.c.
,, 16	20 ,,	,, 11	170 ,,
,, 22	40 ,,	,, 21	Goat bled.

always with the same result. After a period of rest further immunizing doses of plasma were given to the two goats and then their sera were tested again. But again the particular antibody we sought could not be detected. We think it safe to conclude that blood-plasma contains none of that antigen which can give rise to antibody capable of neutralizing tumour filtrate.

The serum of the goat injected with extracts of fowl red blood-cells behaved quite differently. The serum of this goat did contain an antibody capable of neutralizing, to a large extent, the active agent of fowl-grown Fujinami tumour. In order to show this neutralizing power it was essential to add fresh guinea-pig serum ; in the absence of complement no neutralization took place. This antiserum, prepared by injections of extracts of fowl red-cells, had no action at all on filtrate of duck-grown Fujinami tumour, and the result was the same, no matter whether complement were present or not. The following is typical of the experiments carried out with antisera prepared in the manner described already :

On March 22nd, 1932, 8 c.c. of fresh guinea-pig serum was added to 8 c.c. of filtrate freshly prepared from a Fujinami myxosarcoma taken from a fowl. When complement and filtrate had been thoroughly mixed together the mixture was distributed to a series of 4 sterile test-tubes, 3 c.c. to each tube. Each tube received also 3 c.c. of goat-serum which had been heated at 55° C. for 30 minutes, a different serum being used for each tube. The goat-serum used for one tube, A, came from a normal goat ; that for the second tube, B, came from a goat immunized with extracts of fowl embryo ; the serum used for Tube C came from the goat, No. 26, which had been injected repeatedly with fowl plasma ; and the serum used for Tube D, the fourth tube, came from Goat 24, the animal which had been immunized with extracts of fowl red-cells. All four filtrate-serum-complement mixtures were incubated together for 90 minutes at 40° C. and then tested for potency in 4 fowls, each of which received four injections, one from each mixture. In this series of test injections all doses were 1·0 c.c. All 4 fowls gave the same answer. The tumours resulting from injections of the mixture of filtrate, complement and normal goat-serum were similar in size to those resulting from the mixture of filtrate, complement and anti-plasma serum ; all weighed approximately 40 gm. when the fowls died. The tumours resulting from the two remaining mixtures—the mixture containing anti-embryo serum and the one containing anti-serum to blood-cells —were all quite small, varying in size from a small point discovered only by dissection at the site of injection, to a small cluster of tiny tumour masses, which together weighed less than a gramme. Thus all 4 fowls showed that both the anti-embryo serum and the anti-serum to red blood-cells neutralized the tumour filtrate, and they showed, too, that both neutralized to an approximately equal degree.

Additional information concerning the sources and relationships of the anti-tumour antibodies—and consequently of the corresponding antigens—was obtained by means of absorption tests. Emulsions of embryo tissue readily absorb the anti-tumour antibody from anti-embryo serum. This fact, as already mentioned, has been reported in an earlier publication. Absorption was, indeed, to be expected. Would minced embryo absorb the anti-tumour antibody from serum produced by means of extract of red blood-cells ? Tests showed that this antibody was in fact removed quite readily. The operation was easy and the results were definite. Both the anti-erythrocyte and the anti-embryo sera used in the experiment described above, when treated with minced embryo at the same time and in exactly the same manner, lost all their anti-tumour properties. Since the two immune sera were of approximately equal original potency, and since both had been produced readily, this fact that both were absorbed with equal readiness is taken as evidence that the antigent effective in the production of the anti-tumour antibodies in these sera may be identical. This evidence for identity is, however, not as completely satisfactory as could be wished ; the method of comparison is crude, and there is also the fact that minced embryo is not free from blood-cells.

When attempts were made to absorb the anti-tumour antibodies from these same two sera by means of washed red blood-cells considerable difficulty was experienced. Since red blood-cells had provided the antigen used in the production of one of these immune sera, it was expected that red blood-cells would

remove the effective antibody from both, just as minced embryo had done. This expectation was not realized. It proved difficult to remove more than the slightest amount of anti-tumour antibody by means of blood-cells, although the conditions provided were such as had proved adequate with minced embryo tissue. There was little difference between the two sera in this respect. In order to demonstrate the possibility of absorbing any antibody at all, it was found necessary to treat the immune sera at least four times and to extend the process over 4 days. This is the sort of process that was found necessary: To 20 c.c. of serum 3 c.c. of a 50 per cent. suspension of washed blood-cells is added; the mixture is placed in the cold room and is shaken up from time to time; at the end of 24 hours the cells are spun down and removed; the serum is again treated in similar fashion on each of 3 days more. A certain amount of hæmolysis seems unavoidable during these operations—a circumstance which may have modified our experimental results to some extent. When the two immune sera are treated thus thoroughly it is possible to detect a small loss of neutralizing antibody, and in one experiment this loss appeared to be somewhat greater in serum produced with blood-cells than it was in anti-embryo serum. Unequal dilution effects were not the cause of serious experimental errors. The effect of saline unavoidably added to the sera along with the red cells used in the absorptions was controlled. A second volume of immune serum was absorbed in exactly the same way as the first, except that washed duck red cells, which have no effect upon the antibody, were used in place of fowl cells. This duck-absorbed serum was used as the control in all experiments.

The difficulty in absorbing antibody by means of red blood-cells is, perhaps, most simply explained on the assumption that the effective antigen lies inside the blood-cells and cannot come into contact with the antibody in the immune serum, when, as in our experiments, whole red cells are used. Such an explanation is in agreement with the tentative conclusion, based upon the relative efficacy of various methods of extraction, that the infective agent in a tumour filtrate is probably derived from the nucleus of tumour-cells (Gye and Purdy, 1931). Red-cell extracts had been used in the preparation of our immune sera, not whole cells. Even if whole cells had been used, lysis would have taken place in the animal body—a consideration which seems to preclude this method of testing where the effective antigen is situated in the blood-cells. Tests of the absorptive power of lysed cells, undertaken with a similar object, broke down on account of technical difficulties, which at the time we did not know how to overcome.

Fowl blood-cells have been used also in attempts to absorb the neutralizing antibodies peculiar to immune sera prepared by means of tumour extracts. These antibodies are not species specific. An immune serum prepared by injecting a goat with extracts of fowl-grown Fujinami tumours will neutralize filtrate of duck-grown Fujinami tumour. Absorption with minced fowl embryo completely fails to remove this neutralizing power—a fact that has been mentioned already. It has now been shown that blood-cells fail with similar completeness. Representative experiments need not be described.

Attempts to neutralize extracts of the fowl endothelioma M.H.2 by means of sera from goats immunized with fowl red cells proved abortive, because

the filtrates were inactive—a common experience with this tumour. Tumours appeared in only one experiment, and then in only one bird. If we could rely upon the results observed in this one bird, then we could say that an anti-serum to fowl red cells will neutralize the infective agent of a fowl endothelioma. But the evidence, though suggestive, is as yet inconclusive.

We thus arrive at a point where, to facilitate reflection upon the meaning of the facts so far ascertained, it may be useful to set out briefly what to us appear to be the outstanding points concerning the experiments. They are, in tabular form, as follows :

(1) Tumour filtrates have been prepared from fowl-grown or duck-grown Fujinami tumours as necessitated by the experiments.

(2) In filtrates of the Fujinami tumour the infective agent is derived from connective-tissue cells, since the tumour is a myxosarcoma and the infective agent comes from the tumour-cells.

(3) One immune goat-serum was prepared by means of fowl red blood-cells—that is, by means of cells which are not of the same histological class as the cells of the Fujinami tumour. Another serum was prepared by means of fowl plasma.

(4) The immune serum prepared by means of fowl red cells neutralized extracts of fowl-grown Fujinami myxosarcoma.

(5) Although serum prepared by means of fowl blood-cells thus neutralized extract of fowl tumour, the serum prepared by means of fowl plasma did not.

(6) Although extract of fowl-grown tumour was neutralized by serum prepared by means of fowl red cells, extract of duck tumour was not.

We refrain from further discussion. It is clear that as yet we are in possession of isolated facts only. The gaps in our knowledge make it impossible to state with detailed precision the identity, structure and relationships of this substance, which, though derived from body-cells, must be present if the living virus in a tumour filtrate is to infect.

SUMMARY.

In 1931 we published evidence that when a Fujinami tumour passes from fowl to duck, something essential to infectivity changes, since filtrates can no longer be neutralized by immune sera prepared in goats by means of minced fowl embryo.

In the first part of the present paper the reverse process is directly demonstrated by means of serum taken from a goat immunized with minced duck embryo.

In the later parts of the present paper it is shown that serum taken from a goat immunized by means of fowl red cells will neutralize extract of fowl-grown Fujinami tumour. It is shown also that this same immune serum does not affect the potency of extract of duck-grown Fujinami tumour. Serum prepared in a goat by means of fowl plasma is shown not to affect the potency of filtrate prepared from Fujinami tumour, whether fowl or duck ; and the same is shown to be true of serum prepared by means of duck plasma.

REFERENCE.

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THE HETERO-TRANSFER OF TWO FILTERABLE TUMOURS: AN INVESTIGATION BY MEANS OF IMMUNE SERA.

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BOTH the Fujinami myxosarcoma and the Rous sarcoma No. 1 can be transferred from fowls to ducks, but not with equal facility. The Fujinami tumour can be transferred to both young and old ducks, and cell-free extracts and tissue mince are both effective means of transfer. The Rous sarcoma can be transferred only to very young ducklings, and then only by means of minced tissue. The complete failure of cell-free extracts of Rous sarcoma to produce new tumours in ducklings is remarkable because in similar ducklings minced Rous sarcoma tissue not only produces new tumours readily, but produces new tumours which grow freely. The differences between the two tumour strains need elucidation. In both strains a filterable agent can be demonstrated with ease, but the observed differences are not easily explained on the assumption that transfer is a purely infective process. There remains the possibility that when tissue-mince is used the result may be influenced by the survival and multiplication of cells of the inoculum. Clearly more knowledge is needed of how new tumours—both Fujinami and Rous—are formed. The problem was investigated in ducks in a series of experiments of which selected examples have been reported already (Purdy, 1933). The method of inquiry used in these reported experiments depends upon the fact, long familiar to students of transplantable mammalian tumours, that the resistance induced in an animal by an inoculation of embryo tissue is directed solely against the establishment of foreign cells. The conclusions derived from the experiments were: (1) that when minced fowl-grown Fujinami myxosarcoma is injected into an adult duck, the new tumour is formed chiefly or wholly by multiplication of infected host cells (duck cells); (2) that when minced fowl-grown Rous sarcoma No. 1 is injected into a duckling, the new tumour which arises is formed solely by multiplication of cells (fowl cells) of the inoculum; (3) that when minced fowl-grown Fujinami myxosarcoma is injected into a duckling, the new tumour which arises is formed in part by cells (fowl cells) of the inoculum, and in part by multiplication of infected cells (duck cells) of the host. Confirmation or refutation of these conclusions by an independent method is desirable and has been attempted in a series of experiments, of which three representative examples will be described and discussed in the present paper.

The method of analysis which has now been used is a serological one. It is based upon the observations made by Gye and Purdy (1931, 1933) that in the presence of complement an appropriate anti-embryo immune serum will neutralize the infective agent of a tumour filtrate. In the original work tumours produced by cell-free extracts were used. These tumours, developing

solely from infected cells of the host, consisted perforce solely of cells having the same species specificity as the cells of the host. It was observed that a filtrate of fowl tumour is inactivated by an anti-fowl immune serum which has been prepared in a goat by injection of extracts of fowl embryo, but is entirely uninfluenced by an anti-duck serum which has been prepared by injection of extracts of duck embryo. Similarly it was found that a filtrate of duck tumour is rendered inactive by an anti-duck but not by an anti-fowl immune serum. From these observations it follows as a simple corollary that immune sera can be used to determine the species specificity of the tumour-cells from which a given potent cell-free extract has been obtained, for although strictly speaking the species specificity which is revealed by the immune serum is only that of an essential component, the cell element (Gye and Purdy, 1931), of the complex filterable agent, it can hardly be maintained that the species-specificity of the cell element may differ from that of the cell from which the element is derived, and that, for instance, duck cells may conceivably produce substances having the species specificity of fowls. Cell-free extracts capable of producing tumours in fowls can be obtained readily from duckling-grown Rous and Fujinami tumours, hence it follows that the serological reactions can be used to determine whether or not cells of fowl-grown Rous and Fujinami tumours survive and multiply when injected into ducks. Such a determination would test effectively the validity of the conclusions arrived at in the series of experiments, already referred to, in which the resistance induced by an injection of embryo tissue was used as an indicator. The serological method has its limitations, however, when regarded as a general means of investigating the origin of daughter-tumours. It cannot give information when the cells of the inoculum and those of the host belong to one and the same species of animal. It cannot, for instance, give information when cells of a fowl tumour are injected into a fowl. This limitation is due to the fact that in such instances the specific element of the complex tumour agent is identical, no matter whether it be derived from surviving cells of the inoculum or from infected cells of the host.

In the series of experiments to be described in the present paper the method of immunizing the goats by means of embyro extracts, the method of preparing filtrates and the method of carrying out the serological tests have all followed closely the methods adopted in the original experiments of Gye and Purdy (1931), and for this reason need not be described in detail.

Ducks and ducklings in which tumours were produced for use in the tests were all of the Khaki Campbell variety. Fowls were all of the Plymouth Rock variety.

Pulp-sand filtrates alone were used, not candle filtrates. The choice was deliberate. It was obviously highly desirable that all tumour filtrates for the serological tests should be free of cells. Candle filtrates never contain cells; pulp-sand filtrates are less reliable; nevertheless, because of their greater potency, pulp-sand filtrates were preferred and used. The filtrates were made with care, and were, it is believed, always cell-free. But even if occasional viable tumour-cells were present in the filtrates used in the experiments to be described, the conclusions drawn from those experiments would not on that account be invalid. A reduction in the apparent neutralizing power of the immune sera is the only effect which can arise from the presence of viable cells,

and the nature of the experiments is such that a reduction would be of little consequence. Had the effect tended in the opposite direction the possible presence of viable cells in the filtrates would have been a more serious matter.

Three suitable sera were available for the tests; these were (1) a control serum from Goat 6 which was a normal animal; (2) an anti-fowl immune serum from Goat 19; and (3) an anti-duck immune serum from Goat 23. All three goats had been bled on May 4th, 1932. Two days later the sera which had separated from the blood-clots were filtered through candles and then heated for 30 minutes at 55° C. Immunization of Goats 19 and 23 had been carried out in the usual way by means of extracts of whole embryo. The potency and sharp species specificity of the immune sera of Goats 19 and 23 were verified in experiments specially arranged for the purpose, the serum of Goat 6 being used as the normal control. Such verification, however, was hardly necessary in view of the results obtained in the experiments to be described.

The first experiment to which attention is directed is one in which the tumours investigated arose in adult ducks as a result of the injection of minced fowl-grown Fujinami myxosarcoma. The object of the experiment was to find whether such tumours consist wholly of duck cells, the result of a purely infective process, or whether they consist wholly or in part of fowl cells, the result of the survival and multiplication of cells of the injected tissue mince.

On June 24th, 1932, 2 three-month-old ducks, each weighing approximately 1500 gm., were inoculated with a strain of Fujinami myxosarcoma which had been propagated in fowls for many tumour generations. The actual tumour to provide the inocula was of highly active type. A portion of the tumour was minced; the mince was pumped free of air-bubbles, and then 2·0 c.c. of the mince was injected deep into the muscles of the right leg of each duck.

On July 1st, seven days after the minced tissue had been injected, the 2 ducks were killed. In each, at the site of inoculation, there was a large tumour, which on section was found to be soft and fleshy in consistency and deep red in colour. A pulp-sand filtrate was prepared from 5 gm. of tumour tissue—2·5 gm. from each tumour. Then four filtrate-complement-serum mixtures were made in test-tubes. Each mixture contained 1·0 c.c. of filtrate and 1·0 c.c. of fresh guinea-pig serum. In addition each mixture contained 3·0 c.c. of goat serum; but the same goat serum was not used for all the mixtures. That in two mixtures was the normal serum of Goat 6; that in another was the anti-fowl serum of Goat 19; and that in the remaining mixture was the anti-duck serum of Goat 23. The four mixtures thus prepared were incubated at 37° C. for 30 minutes in order to give time for the goat sera to act. Then samples of the mixtures were injected into fowls to test whether either immune serum had reduced the potency of the portion of filtrate with which it had been mixed. For the fowl injections all doses were 1·0 c.c. The birds used, the mixtures given, the sites injected and the results observed are all shown in Fig. 1.

The anti-fowl serum failed to affect the potency of the filtrate; the anti-duck serum neutralized to a very considerable extent. From these facts, which have been confirmed in other experiments not here reported, it is concluded that when minced fowl-grown Fujinami myxosarcoma is injected into

large ducks, the resulting tumour arises wholly from infected host-cells, and not at all by multiplication of cells of the inoculum. This conclusion is in complete agreement with that drawn from observations made upon ducks injected with embryo tissue and reported in the paper already referred to. The failure of the anti-duck serum to neutralize the filtrate completely does not invalidate the conclusion drawn from the experiment. In our experience anti-embryo sera have rarely been sufficiently active to neutralize completely a highly potent tumour filtrate.

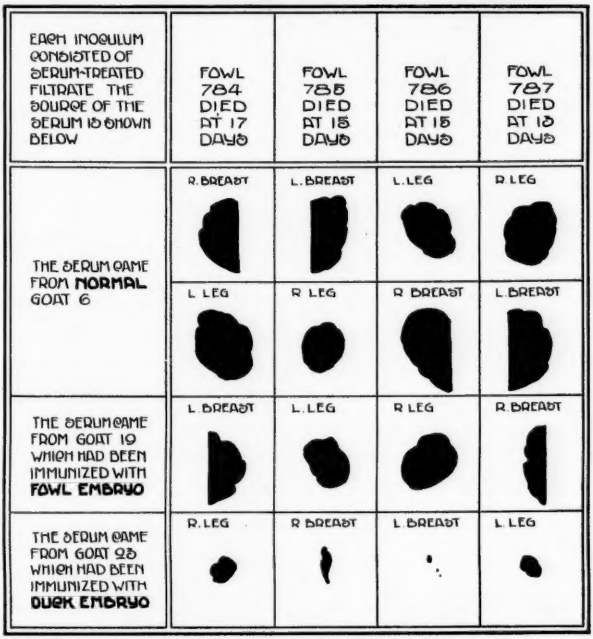
EACH INOCULUM CONSISTED OF SERUM-TREATED FILTRATE THE SOURCE OF THE SERUM IS SHOWN BELOW
FOWL 784 DIED AT 17 DAYS
FOWL 785 DIED AT 15 DAYS
FOWL 786 DIED AT 15 DAYS
FOWL 787 DIED AT 15 DAYS
THE SERUM CAME FROM NORMAL GOAT 6
R. BREAST
L. BREAST
L. LEG
R LEG
L LEG
R LEG
R BREAST
L BREAST
THE SERUM CAME FROM GOAT 19 WHICH HAD BEEN IMMUNIZED WITH FOWL EMBRYO
L. BREAST
L. LEG
R LEG
R BREAST
THE SERUM CAME FROM GOAT 23 WHICH HAD BEEN IMMUNIZED WITH DUCK EMBRYO
R. LEG
R BREAST
L BREAST
L LEG

FIG. 1.—The filtrate was derived from a tumour which followed the injection of minced fowl-grown Fujinami myxosarcoma into an adult duck. Anti-duck immune serum neutralized, but anti-fowl serum did not.

The serological method has been used also to investigate the origin of daughter-tumours which develop when minced fowl-grown Rous sarcoma No. 1 is injected into ducklings. The tests carried out by means of the resistance induced in ducklings by an inoculation of fowl embryo, and already reported, led to the conclusion that no matter for how many generations the Rous sarcoma is propagated in ducklings the tumour-cells remain fowl-cells. Confirmation or refutation of this conclusion was particularly desirable. The results obtained by the serological method are illustrated in two experiments which will be described now. It will be seen that, except for the source of the tumour filtrates and the results of the test inoculations in fowls, both experiments were duplicates of that summarized in Fig. 1.

To provide tumours for the tests a strain of Rous sarcoma was propagated in ducklings for six tumour-generations by means of 0·5 c.c. doses of minced tumour-tissue. The ducklings were all very young. One was seven days old at the time it was used; not one of the others was more than four days.

The first duck-generation of the series was started on April 18th, 1932, when a 4-day duckling was inoculated with minced fowl-grown Rous sarcoma No. 1. The series was brought to an end on May 28th, when the two ducklings

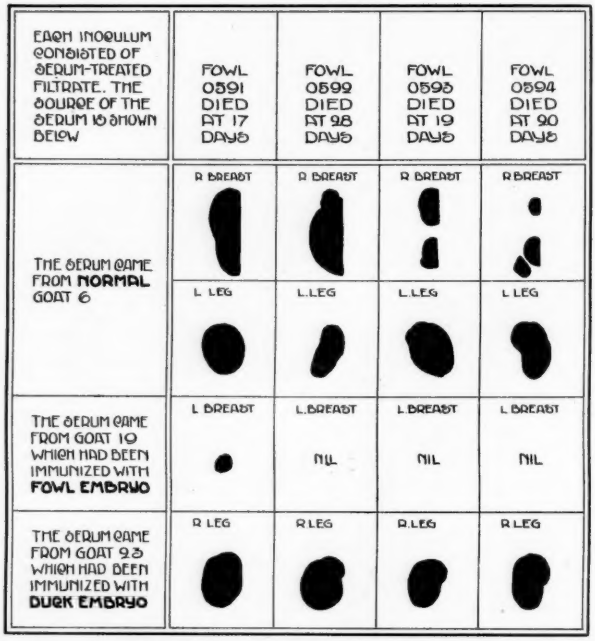
EACH INOCULUM CONSISTED OF SERUM-TREATED FILTRATE. THE SOURCE OF THE SERUM IS SHOWN BELOW
FOWL 0591 DIED AT 17 DAYS
FOWL 0592 DIED AT 28 DAYS
FOWL 0593 DIED AT 19 DAYS
FOWL 0594 DIED AT 20 DAYS
THE SERUM CAME FROM NORMAL GOAT 6
R BREAST
L LEG
THE SERUM CAME FROM GOAT 19 WHICH HAD BEEN IMMUNIZED WITH FOWL EMBRYO
L BREAST
NIL
THE SERUM CAME FROM GOAT 23 WHICH HAD BEEN IMMUNIZED WITH DUCK EMBRYO
R LEG

FIG. 2.—The filtrate was derived from a Rous sarcoma of the fourth generation in ducklings. Anti-fowl serum neutralized, but anti-duck serum had no effect.

carrying the sixth tumour generation were killed. Only experiments made with tumours of the fourth and sixth generations will be described. Experiments made with tumours of earlier generations gave similar results.

The experiment in which the tumour of the fourth duck-generation was investigated was carried out on May 17th, 1932. Only one tumour was available. The experiment in which the sixth duck-generation was investigated was carried out on May 28th. On this occasion two tumours were available, and both were used in the preparation of the pulp-sand filtrate which was to be tested by means of the immune sera. In both these experiments with duck-grown Rous sarcoma four filtrate-complement-serum mixtures were made up as usual. Each mixture contained 1·0 c.c. filtrate, 1·0 c.c. fresh

guinea-pig serum and 3·0 c.c. of goat serum. As usual, also, in two of the mixtures the goat serum was that of Goat 6, the normal animal; in one mixture it was the anti-fowl serum of Goat 19; and in the remaining mixture it was the anti-duck serum of Goat 23. In both experiments, too, the four mixtures were incubated at 37° C. for 30 minutes in order, as before, to give each immune serum time to act upon the portion of filtrate with which it had been mixed. To test the potency of the different incubated mixtures, samples were injected into fowls. All doses were 1·0 c.c. All other essential details

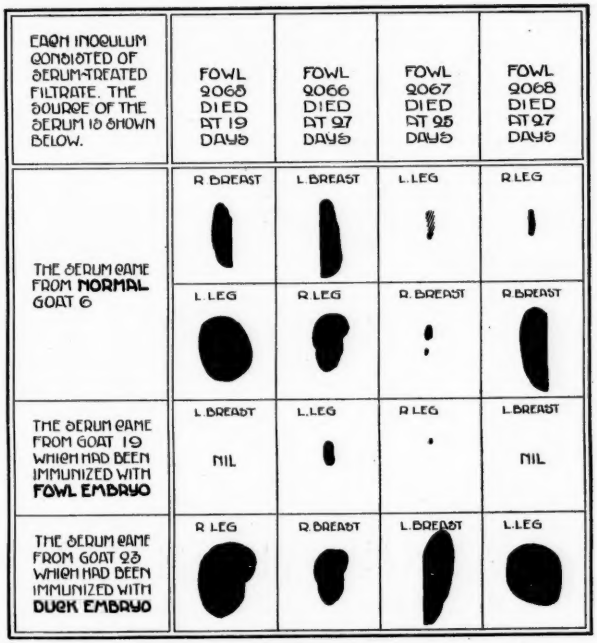
EACH INOCULUM CONSISTED OF SERUM-TREATED FILTRATE. THE SOURCE OF THE SERUM IS SHOWN BELOW.
FOWL 2065 DIED AT 19 DAYS
FOWL 2066 DIED AT 27 DAYS
FOWL 2067 DIED AT 25 DAYS
FOWL 2068 DIED AT 27 DAYS
THE SERUM CAME FROM NORMAL GOAT 6
R. BREAST
L. BREAST
L. LEG
R. LEG
L. LEG
R. LEG
R. BREAST
R. BREAST
THE SERUM CAME FROM GOAT 19 WHICH HAD BEEN IMMUNIZED WITH FOWL EMBRYO
L. BREAST
NIL
L. LEG
R. LEG
L. BREAST
NIL
THE SERUM CAME FROM GOAT 23 WHICH HAD BEEN IMMUNIZED WITH DUCK EMBRYO
R. LEG
R. BREAST
L. BREAST
L. LEG

FIG. 3.—Same as Fig. 2, except that the filtrate was prepared from a Rous sarcoma of the sixth generation in ducklings.

of the potency tests, including the results observed, are shown in the accompanying figures. Fig. 2 summarizes the serological test of filtrate prepared from the tumour of the fourth duck-generation, and Fig. 3 summarizes the exactly similar experiment made with filtrate of the sixth generation.

It will be seen that in both experiments the results were the same. The filtrates of duck-grown Rous sarcoma No. 1 were unaffected by the anti-duck serum, but were very considerably reduced in potency by the anti-fowl serum. The neutralization by the anti-fowl serum proved the survival of fowl cells—cells which could only have been derived from the tumour-cells inoculated into the first duckling of the series; the complete failure of the anti-duck serum to

neutralize showed that duckling cells had not become infected, and yet for forty days the sarcoma had been growing in ducklings, and in that time had reached its sixth tumour-generation. Thus it was shown that here in the duckling strain of the Rous sarcoma there is a genuine example of a malignant tumour-cell which is living a parasitic life. The duckling host is not only a different individual from that in which the tumour-cells originated, but it actually belongs to a different species of animal. The experiments show that the Rous sarcoma growing in ducklings is the result not of infection of host-cells, but wholly of the survival and multiplication of cells of the inoculum. This is exactly the conclusion which was arrived at as the result of experiments, already reported, in which ducklings injected with minced embryo tissue were used to indicate whether or no cells of the inoculum play a direct part in the production of daughter-tumours.

Thus two of the conclusions arrived at by the embryo-resistance method have been confirmed completely. Confirmation of the third conclusion—the one relating to the mode of origin of tumours which follow upon the injection of minced fowl-grown Fujinami myxosarcoma into ducklings—has not been attempted. It seemed unnecessary. The main object of the serological investigations was to find whether the results of the experiments reported in the previous paper (Purdy, 1933) will bear the interpretation placed upon them. This object had been attained.

It will have been noticed how completely it has been taken for granted that the tumour-cells themselves and not normal cells are the source of the species-specific substance which is an essential constituent of the infective tumour agent. Justification seems hardly necessary. The inactivation of a cell-free extract of a Rous sarcoma of the sixth duckling-generation, as in the experiment which is illustrated in Fig. 3, admits of only one explanation, and that a fairly obvious one. If it be supposed that the fowl-specific substance which is neutralized in such an experiment does not arise in the tumour-cells, there are but two alternatives : the substance may be supposed to arise either in normal host-cells, which are duck-cells, or in transplanted normal fowl-cells. Both alternatives are untenable. That duckling cells should produce substances having the species specificity of fowl-cells could be dismissed on general grounds, as already indicated, even if it were not already known (Gye and Purdy, 1931, 1933) that duck cells which have been infected by filtrates of fowl-grown Fujinami myxosarcoma never yield filtrates which are affected by anti-fowl immune serum. That the neutralizable substance arises in transplantable normal fowl-cells which accompany the tumour-cells is also a suggestion which can be dismissed as entirely improbable, since it is known from the work which Russell and others have done in connection with transplantable mammalian tumours that injected stroma and other normal cells soon die, even when they are derived from an animal of the same species as the new host. Even cells of fowl embryos are not transplantable in ducklings beyond the first, or perhaps the second generation. Thus in these experiments with a duckling strain of the Rous sarcoma No. 1, there is direct proof that the cell-element of the complex infective agent arises in the tumour-cells themselves. On less direct evidence Gye and Purdy (1931) came to a similar conclusion. They tested normal cells and found good reason for concluding that these

cannot be the source of the neutralizable specific substance which forms an essential part of an infective cell-free extract of the Fujinami myxosarcoma. The accurate determination of the source of these specific substances has an importance in connection with discussions of the relationship which subsists between these substances and the associated tumour viruses.

SUMMARY.

A serological method dependent upon the published observations of Gye and Purdy has been used in a re-investigation of the way in which daughter-tumours are formed when tissue from fowl-grown Rous and Fujinami tumours is injected into ducks.

The new method shows that in adult ducks new Fujinami tumours arise solely because cells of the host become infected and multiply. It shows also that in ducklings the Rous sarcoma No. 1 is propagable solely because the tumour-cells of the inoculum become established and multiply; no infection of host-cells takes place. Thus by an independent method conclusions drawn from observations made upon ducklings which had received an injection of embryo tissue have been confirmed.

Gye and Purdy (1931) concluded that the species-specific element which is present in an active tumour filtrate and is necessary for infectivity must be derived from the tumour cells themselves, and not from normal tissues of the host. The accuracy of this conclusion is proved by new facts.

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OBSERVATIONS ON THE DEVELOPMENTAL FORMS OF PSITTACOSIS VIRUS.

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A MORPHOLOGICAL study of the virus of psittacosis has shown that the elementary body is not the only form assumed by this virus (Bedson and Bland, 1932). In the early stages of development the virus occurs in the form of large, apparently homogeneous masses, to which the term "plaque" has been given. Later the virus masses are no longer homogeneous, but are seen to be composed of a number of more or less equal forms 1μ or more in

greatest diameter—" large forms "—and these large forms by repeated division give rise again to the elementary bodies. The term " morula " was used to denote the composite virus masses, and the qualifications " primary ", " intermediate " and " elementary body " to indicate the size of the component particles. Repeated observations showed that these forms followed each other in regular sequence, forty-eight hours being required in the mouse for elementary bodies to pass through these various forms and back again to elementary bodies. And with a strain of high and fixed virulence the regularity with which this sequence of forms occurred was such that one could predict with certainty the form in which the virus would be found in the spleen of a mouse killed at any given time after infection. This led to our putting forward the view that the virus of psittacosis passed through a developmental cycle (Bedson and Bland, 1932). Two hypothetical explanations of this cycle were advanced. One assumed that the plaques were in truth homogeneous—plasmodia—and the virus therefore akin to the protozoa ; the other claimed only apparent homogeneity for the plaques. In the latter case the virus would be a minute bacterium, which in the early stages of multiplication produced abnormally large forms, secreting a mucoid material which ensured the cohesion of the colony and obscured its detail. Although we declared a preference for the former explanation, it was recognized that both were merely hypotheses and the evidence entirely morphological. The study of these developmental forms of psittacosis virus has therefore been pursued in the hope of obtaining a further insight into their meaning and nature, and the findings form the subject of this paper. For the sake of brevity, and to avoid confusion, the terms " plaque ", " morula ", " large forms ", etc., will be retained, but in doing so the possibility of having to reconsider this nomenclature has not been lost to view.

VIRULENCE OF THE DIFFERENT FORMS.

It was thought that the virulence of the various developmental forms might throw some light on the problem. Should this virus be a bacterium, then one would not necessarily expect the early stages to differ markedly in their virulence from the elementary bodies, whereas if it were a protozoan, the infective and virulent stage would probably be represented by the elementary body. Mice were inoculated intraperitoneally with suspensions of virulent spleen in which the virus was present in the form of elementary bodies. Large doses—0·5 c.c. of a 5 per cent. spleen suspension—were used in order that there should be ample virus present in the infected mouse for morphological study. Mice were killed at varying intervals after infection, and their spleens removed and washed several times in a large excess of saline to remove any adherent virus. A portion was then snipped off wherewith to make impression preparations, and the remainder suspended in phosphate buffer to give a 5 per cent. suspension. A portion of this suspension was centrifuged for one hour at 5000 r.p.m., and smears made of the deposit were stained by Giemsa and Castaneda and examined for the presence of elementary bodies. Another portion was used for carrying out a virus titration in the guinea-pig's skin, whilst the remainder was used for testing the virulence of mice. Two mice were inoculated with

each suspension, the dose being 0·5 c.c. intraperitoneally. It is recognized that the identification of elementary bodies when they are present in small numbers, even when concentration by the centrifuge is resorted to, is a matter of extreme difficulty. In the case of psittacosis virus, however, one is helped by the large size of the elementary body, and the fact that with Castaneda's stain they are blue, whereas cell *débris* take the safranin counter-stain. Even so it would be impossible to speak with certainty of their absence. The findings in these experiments are recorded in Table I.

Several features of this table call for comment. One is the rapidity with which the virus reaches the spleen. Spleens removed 1½ hours after inoculation have always proved virulent, and though no virus could be identified in the imprint preparations, elementary bodies were present in the centrifuge deposits. The second point is that between 5 to 15 hours after inoculation elementary bodies can no longer be found in the suspensions, and that the virulence of these spleens is so low that the mice inoculated with them either survive or die after a long incubation period. Taking the 5 spleens in which elementary bodies could not be found, it will be seen that 4 of the inoculated mice survived and 5 died of virus infections the period between inoculation and death being invariably long. On the other hand, wherever elementary bodies were undoubtedly present the spleen suspension has proved virulent. This absence of elementary bodies and low virulence corresponds with the presence of plaques and early morulæ in the spleen, and suggests that the virus in this form is either of low virulence or avirulent. Against this interpretation it might be argued that the plaques and large forms have nothing to do with psittacosis virus, which throughout maintains the form of elementary bodies, and that the inoculated virus is in major part destroyed, the surviving portion multiplying and appearing again in appreciable quantity somewhere about the 15th hour after inoculation. It is true, too, that the majority of these spleen suspensions of low virulence give only poor titres in the guinea-pig's skin. Such a point of view, however, is untenable for several reasons. A spleen suspension containing virus in the form of elementary bodies can be diluted far beyond the point at which it will give a reaction in the guinea-pig's skin and still prove virulent for mice. A 5 per cent. suspension of an elementary body spleen will give a titre of 10^{-4} in the guinea-pig's skin, whereas in the mouse the 10^{-7} or even 10^{-8} dilution may still prove fatal. However, all the spleen suspensions of low virulence gave reactions in the guinea-pig's skin; some even, such as the one made from the mouse M. 833, had a titre of 10^{-2} comparable to the titre given by the 24-hour spleen suspension M. 740, which killed mice in 3 days. Then, again, the very high dilutions of suspensions of elementary bodies which are infective for mice suggest that, with this strain of high virulence, the introduction of only a few elementary bodies suffices to set up a fatal infection; this is hardly in keeping with the view that a major portion of the virus introduced is destroyed. It would appear, then, that the elementary bodies introduced into the peritoneal cavity reach the spleen and there produce the plaques, which about the 12th to 14th hour change into morulæ of large forms. These latter by division give rise to elementary bodies, which begin to appear again between the 15th and 24th hour. Similarly it would appear that the virus in

TABLE I.—*The Virulence of the Various Developmental Forms of Psittacosis Virus.*

Time of removal of spleen after inoculation.	Mouse.	Microscopic examination. Spleen imprint.	Microscopic examination. Presence of elementary bodies in centrifuge deposit.	Titration of virus. 1/1.	1/10.	1/100.	1/1000.	1/10,000.	Virulence for mice.	
1½ hours	M. 805	No virus seen	Present (±)	+	±	?	−	−	D. 8	D. 8.
	M. 816	,, ,,	,, (±)	+	±	−	−	−	D. 7	D. 7.
	M. 830	,, ,,	,, (±)	±	∓	−	−	−	D. 8	D. 10.
5 ,,	M. 806	Few early plaques	,,	+	±	∓	−	−	D. 4	D. 7.
	M. 817	,, ,,	,,	+	∓	−	−	−	D. 7	D. 7.
	M. 831	,, ,,	?	±	?	−	−	−	D. 10	S.
8 ,,	M. 739	Plaques	?	±	∓	−	−	−	D. 5	D. 6.
	M. 752	,,	None seen	±	?	−	−	−	S.	S.
12 ,,	M. 764	Plaques and a few large forms	,, ,,	±	∓	−	−	−	D. 9	S.
14 ,,	M. 766	Plaques and large forms	,, ,,	±	±	−	−	−	D. 9	D. 9.
15 ,,	M. 767	Plaques	,, ,,	+	±	−	−	−	D. 11	S.
	M. 818	Plaques and large and intermediate forms	Present (∓)	++	+	±	−	−	D. 5	D. 7.
	M. 833	Plaques and large forms	None seen	+	±	∓	−	−	D. 3*	D. 10.
24 ,,	M. 740	Large and intermediate forms and E.B.'s	Present (+)	+	±	∓	−	−	D. 3	D. 3.
	M. 753	Ditto	,, (+)	++	+	±	?	−	D. 3	D. 4.
	M. 832	,,	,, (+)	+	±	?	−	−	D. 4	D. 4.
48 ,,	M. 741	Elementary bodies	,, (++)	+	±	∓	?	−	D. 2	D. 2.
	M. 754	,, ,,	,, (++)	++	+	±	∓	−	D. 2	D. 2.

S. = Survived. D. = Died. The number indicates the day after inoculation when death occurred.
* Died from bacterial infection.

the form of plaques and primary morulæ is of low virulence for the mouse—incomparably so when compared with virus in the form of elementary bodies—but is capable of producing a reaction in the guinea-pig's skin. The guinea-pig's skin would seem to be more a test for virus *qua* virus rather than of the form it is in.

SEROLOGICAL REACTIONS WITH SPLEENS CONTAINING LARGE FORMS.

It is possible, of course, that the reactions given by the guinea-pig's skin to suspensions of low virulence for the mouse are not reactions to psittacosis virus, but to some other virus present in the mouse spleen which is lit into activity by the psittacosis infection. In order to see whether this were so or not, neutralization and complement-fixation tests were carried out with an anti-psittacosis serum and a suspension of mouse spleen containing virus in large forms, but no demonstrable elementary bodies. The antiserum had been prepared in the guinea-pig with guinea-pig virus. As will be seen from Table II, this spleen suspension not only gave good complement-fixation with the specific serum, but was neutralized by it.

STUDY OF DEVELOPMENTAL FORMS OF PSITTACOSIS VIRUS IN OTHER SITES THAN THE MOUSE SPLEEN.

In previous work the development of the virus had been followed in mouse spleen either *in vivo* or in tissue culture. Since then it has been studied in the liver of mice and in the spleen of guinea-pigs. In the mouse liver the appearances observed corresponded very closely with what had been observed in the spleen. There was the same succession of forms, and in the same animal the picture presented by the virus in the spleen was mirrored in the liver. As has been pointed out before (Bedson, 1933), the guinea-pig is much less susceptible to this virus than is the mouse. Nevertheless some multiplication of the virus does take place in this animal, although it rapidly disappears again. Four guinea-pigs were inoculated intraperitoneally with a large dose of virus (2·0 c.c. of a 5 per cent. spleen suspension), and then killed at intervals of 5, 12, 36 and 48 hours after inoculation. Imprint preparations were made from the spleen and stained by Giemsa and Castaneda for morphological study, and a 10 per cent. suspension was prepared for titration in the guinea-pig's skin. The titration showed evident multiplication, which was at its height 36 hours after inoculation, as the following data show:

Guinea-pig.	Time after inoculation when killed.	Temperature.		Virus titration.			
		Before inoculation.	When killed.	1/1.	1/10.	1/100.	1/1000.
P. 209	5 hours	102·0° F.	106·0° F.	±	—	—	—
P. 206	12 ,,	101·4° F.	105·6° F.	∓	—	—	—
P. 207	36 ,,	103·0° F.	105·2° F.	++	+	±	?
P. 208	48 ,,	102·8° F.	104·8° F.*.	±	∓	—	—

* Had risen to 106·1° F. 36 hours after inoculation.

Microscopical examination of the spleen preparations gave the following results: No virus was seen in P. 209. In P. 206 there were small plaques

TABLE II.—*Neutralization and Complement-fixation with a Sixteen-hour Spleen.*

Antigens { M. 954/5. 5% suspension of mouse spleen containing virus in the form of elementary bodies.
M. 956/7. 5% suspension of mouse spleen containing virus in large forms only (16-hour spleen).

Sera { P. 112. Anti-psittacosis serum made in the guinea-pig.
N.G.P. Normal guinea-pig serum.

NEUTRALIZATION.

Virus dilutions and undiluted serum mixed in equal parts and inoculated I. D. in the guinea-pig in a dose of 0·2 c.c. after 2 hours at room temperature.

	Final dilutions of virus. 1/2.	1/10.	1/100.	1/1000.
Virus suspension M. 956/7 + N.G.P. serum	+	±	∓	−
Virus suspension M. 956/7 + serum P. 112	±	−	−	−

COMPLEMENT-FIXATION.

Fixation for 2½ hours at room temperature. Two readings taken, one after 30 minutes at 37° C. and the other after standing overnight at room temperature.

Serum.		Antigens + 2 M.H.D. complement. M. 954/5.	M. 956/7.	Saline.
P. 112	1/4	++++	++++	−
		++++	+++	−
	1/8	++++	+++	..
		+++	++	..
	1/16	++++	++	..
		++	±	..
	1/32	+++	±	..
		+	−	..
	1/64	++	−	..
		−	−	..
N. G. P.	1/4	−	−	−
		−	−	−
Saline		−	−	−
		−	−	−

and some primary morulæ; none, however, reached the size of those observed in the mouse. The virus in the preparations made from spleen P. 207 was mostly in the form of elementary bodies, but some intermediate and occasional large forms were seen. In P. 208, apart from a rare mononuclear cell containing scattered elementary bodies, no virus was seen. The impression gained was that the virus introduced was in part destroyed, and that the surviving portion multiplied, only to be destroyed in its turn. However, developmental forms similar to those seen in the mouse could be identified.

FILTERABILITY OF THE LARGE FORMS OF PSITTACOSIS VIRUS.

If the large forms seen in the microscopical preparations are in fact psittacosis virus, then one would expect the filterability of virus in this form to differ from that of virus in the form of elementary bodies. These experiments have been made with spleens removed 16 to 18 hours after inoculation, and in every case the morphology of the virus was studied to ensure that it was present as large and intermediate forms, but had not progressed as far as elementary bodies. The filterability of these suspensions was always compared with the filterability of an elementary body suspension. In the first three experiments Berkefeld V candles were used, and in order to minimize, as far as possible, the factor of candle variability, in experiments 1 and 2 a pair of candles was used, and their order reversed in the two experiments, so that each candle was used for both types of virus suspension. In the third experiment one candle was used throughout, the large-form suspension being filtered first. Another factor which had to be controlled was the varying richness in virus of the different suspensions, and in order to overcome this, all the suspensions were titrated in the guinea-pig's skin, each pair of one experiment in the same animal, and the necessary adjustment made by dilution to bring them approximately to the same titre. The findings of these experiments are given in Table III.

In experiment 2 both the mice and in experiment 3 one of the two receiving the large-form filtrate died. In the spleens of the two dying in the former experiment no virus was seen on microscopical examination, but no cultivable bacteria were present either. In the latter experiment the same was true of the mouse which succumbed, and in this case passage to further mice was negative. It is difficult to account for the death of these three mice, and the possibility that it was due to a small dose of virus cannot be ruled out. All one can say is that it is most exceptional for a mouse to die of psittacosis and to be unable to demonstrate virus in its spleen. The remaining mice receiving the large form filtrates in these experiments all survived, whilst those inoculated with the elementary body filtrate all died, and the presence of virus in their spleens was verified microscopically. Thus it would appear that the virus present in suspensions of early spleens (large and intermediate forms) filters less readily than virus in the form of elementary bodies.

CENTRIFUGE EXPERIMENTS.

It was thought, too, that the relative centrifugability of the virus at different stages of development might throw light on the correctness of the interpretation

TABLE III.—*Filterability of the Large Forms of Psittacosis Virus.*

Experiment.	Material filtered. 5% phosphate suspension of spleens removed.	Morphology of virus.	Filter.	Virulence for mice. Unfiltered.		Filtered.		Remarks.
1	18½ hours after infection	Large and intermediate forms	Berkefeld V¹	D. 5	D. 6	S.	S.	
	46 ,, ,, ,,	Elementary bodies	,, V²	D. 4	D. 5	D. 7	D. 7.	
2	17 ,, ,, ,,	Large and intermediate forms	,, V²	D. 4	D. 5	D. 7	D. 17	No virus found in the spleens of mice which died after receiving the 17-hr. filtrate. No cultivable bacteria present. Passage not carried out.
	41½ ,, ,, ,,	Elementary bodies	,, V¹	D. 5	D. 5	D. 3	D. 5	
3	16 ,, ,, ,,	Large and intermediate forms	,, V²	D. 6	D. 6	D. 8	S.	No virus present in the spleen of mouse which died after receiving the 16-hrs. filtrate. Passage negative.
	3 days ,, ,,	Elementary bodies, some large and intermediate forms	,, V²	D. 3	D. 3	D. 17	D. 17	
4	16 hours ,, ,,	Large forms and a few intermediate forms	Chamberland L. 1 *bis*	D. 8	D. 8	S.	S.	The mouse dying 2 months after inoculation with 4-day filtrate became ill at the same time as its fellow, and remained ill throughout. Virus present.
	4 days ,, ,,	Elementary bodies	Ditto	D. 3	D. 3	D. 7	D. 2	

TABLE IV.—*Centrifugation of Large Forms and Elementary Bodies.*

Suspension. Time of removal of spleen after infection.	Morphology of virus.	Centrifugation.	Titration of virus. Before centrifugation. 1/1.	1/10.	1/100.	1/1000.	1/10,000.	After centrifugation. 1/1.	1/10.	1/100.	1/1000.	1/10 000.
17 hours	Large forms	3500 r.p.m. 45 min.	0	+	±	−	−	+	±	−	−	−
48 ,,	Elementary bodies	,, ,,	0	++	++	+	±	+	±	−	−	−
14 ,,	Large forms	4500 r.p.m. 1 hour	+	∓	−	−	−	±	?	−	−	−
48 ,,	Elementary bodies	,, ,,	0	++	+	±	∓	0	∓	−	−	−

of our morphological studies. It was imagined that the large forms would sediment more readily than the elementary bodies. The findings of two experiments summarized in Table IV show that this is not so.

In both cases the deposition brought about by centrifugal force is greater in the case of the elementary body suspension, and one can only conclude that the large forms are much less dense than the elementary bodies, and that the greater surface presented by the former to the currents set up by centrifugation interferes with their being thrown down.

SUPRAVITAL STAINING.

Cowdry (1922) has shown that the inclusion bodies of vaccinia can be stained supravitally with such stains as cresyl blue and neutral red in high dilution. Minute portions of the spleen of a mouse were teased out on a slide in a drop of saline containing 1 in 10,000 cresyl blue, mounted with a cover-slip and ringed with vaseline. Examined under the microscope, such preparations show a considerable number of cells containing more or less circular blue staining inclusions in their cytoplasm. These inclusions stain intensely with the blue and vary in size; they stain almost immediately, and lose their stain again in about 30 minutes owing to reduction of the dye. It is recognized that all blue-staining material in such a preparation is not virus, but comparison with preparations made from normal spleen show that blue-staining masses in the psittacosis preparations are larger and in much greater number. Further, if in an unmounted preparation observed with a low power a cell containing a blue inclusion is marked down, and when dry decolorized and stained for virus, it will be found that the majority of the blue-staining masses are virus colonies. A similar conclusion is arrived at if a mounted preparation is viewed first with direct illumination, and a suitable cell having been located in the centre of the field, examined then with the dark ground; the virus particles can be made out. Not only do these blue-staining inclusions occur inside the cell, but some will be found free in the surrounding fluid, and when inside the cell they can sometimes be freed by tapping on the cover-slip. Not all these blue-staining inclusions appear homogeneous; some show apparent structure. Cowdry (1922) made the same observation in the case of vaccinia. But since a spleen containing practically all the virus in the form of elementary bodies may show just as many homogeneous blue-staining inclusions as an early spleen, it would seem that the apparent structure has no relation to virus particles. The blue stain is dispersed by 1 per cent. acetic acid run under the cover-slip, but some material remains at the site of the inclusion which is of lower refractivity than chromatin. These inclusions are not blackened by osmic acid. It has not been possible by these means to settle the question of whether the plaques are homogeneous or not. There still seem to be some blue-staining masses in early spleens, which under dark-ground illumination appear homogeneous. And if preparations made from a spleen containing virus in the from of plaques and primary morulæ be treated with 5 per cent. acetic acid when dry, but unfixed and then fixed in methyl alcohol and stained with Giemsa, although the details in the primary morulæ are clearer, there still appear to be homogeneous masses present. The reason why these supravital staining experiments have been referred to in detail is that they point to psittacosis being a

virus which gives rise to an inclusion body. In the accepted sense of the term " psittacosis " does not produce inclusions. Nevertheless the colonies of this virus would appear to be enveloped in some material which, like the vaccinia inclusion, stains supravitally with cresyl blue, but unlike vaccinia is not acidophil in fixed preparations. Affinity for acid dyes, though a common feature of virus inclusions, is not an essential one ; typical inclusions like those of herpes may be basophilic at certain stages. Then again a virus-like ectromelia, though producing excellent acidophilic inclusion bodies in the skin, fails to do so in the liver or spleen (Marshall, 1930), yet imprint preparations made from the liver or spleen of a mouse dead of ectromelia show undoubted cytoplasmic colonies of the virus.* The suggestion is made, therefore, that there is not necessarily any real difference between the viruses which produce typical inclusion bodies and those which do not ; the difference is a quantitative rather than a qualitative one. In the case of psittacosis virus the inclusion lacks the density of those produced by vaccinia, fowl-pox or ectromelia growing in skin ; all the details of the virus colony can be seen through the tenuous covering. At any rate this is true of the later stages of development of the psittacosis colony. What one cannot be certain of is that the homogeneity of the early psittacosis colony is not merely apparent due to the greater quantity and density of inclusion material, which becomes adsorbed as the colony ages, revealing the colonial structure with greater and greater clearness. In other words it has not been possible to decide whether the affinities of this virus are protozoal or bacterial. There is one point, not so far touched on, which might have thrown light on this problem. That is the richness in plaques of the early spleen. Were this virus a protozoan, then one would expect a spleen removed, say, 14 hours after infection and containing virus in the form of plaques to contain approximately the same bulk of virus as the 48-hour spleen, where all the virus has divided up into elementary bodies. Unfortunately this is a point on which it is difficult to pronounce categorically, because the plaque is not always easy to identify with certainty. In the case of extracellular material in imprint preparations, it has often been found impossible to distinguish between plaques and broken-off portions of basophilic cytoplasm ; only when the plaque occurs in the cytoplasm of an intact cell can one feel sure, and many cells get disintegrated in making the preparations.

CONCLUSIONS.

1. Evidence has been produced to show that the forms described in the early stages of infection of the mouse with psittacosis are developmental stages of the virus.

2. The virulence of the virus in the form of plaques or primary morulæ is considerably less than that of the elementary bodies.

3. In the large or intermediate forms it filters less readily than the elementary bodies, but the latter are thrown down more readily by centrifugation.

4. It would appear that psittacosis virus produces inclusions, though not the fully developed acidophil inclusion characteristic of many viruses.

* I am indebted to Capt. S. R. Douglas, F.R.S., for kindly providing me with a strain of ectromelia virus.

5. It has been found impossible to say whether the plaque is homogeneous —a plasmodium—or an aggregate of virus particles whose detail is obscured by inclusion material.

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THE REVERSIBILITY OF NEUTRAL SALT ACTION ON COMPLEMENT.

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IN a recent publication (Gordon and Thompson, 1933) the writers referred to the generally accepted view that the inhibition of a complement by neutral salts is reversible on dilution. As the literature, however, offers little exact information on this point, it has been considered worth while to examine this reversibility, and particularly to find whether it is entire and complete or only partial.

The method of experiment was as follows: An inhibitory dose (I.D.) of neutral salt was added to 1 c.c. of guinea-pig serum.

The minimum inhibitory doses (M.I.D.) expressed as concentrations of salt in the salt-serum mixture are approximately as follows: Chloride, 0·4–0·5 N; bromide and nitrate, 0·3–0·4 N; iodide, 0·25–0·35 N; thiocyanate, 0·2 N. (Gordon and Thompson, 1933). The dose of salt used in the first experiment was in each case rather less than twice the M.I.D., except in the case of iodide, for which an additional test was set up, using a concentration of salt only just in excess of the M.I.D.

The salt-serum mixture was then allowed to stand for two hours. A test was then made to ensure that complete inhibition had taken place. After standing, the mixture was then diluted with saline sufficiently to reduce the salt concentration to a point below the inhibition level. After allowing to stand for a further hour, tests were made to find to what extent complement activity had returned (reversibility tests).

Two sets of controls were set up. In the first set the serum was diluted with saline *before* adding the same I.D. of neutral salt as was used in the reversibility

tests. The second set of controls may be described as "volume controls". The serum was treated exactly as in the reversibility tests, except that saline was used instead of the neutral salt solution, as well as for further dilution.

All mixtures were tested for complement activity by adding 0·05 and 0·1 c.c. to 0·5 c.c. of a 5 per cent. suspension of sensitized ox cells.

The results with sodium salts are given in Table I. The extent of hæmolysis is given as follows: 4, complete hæmolysis; 3, nearly complete; 2, partial; 1, trace of hæmolysis; 0, indicates no hæmolysis.

Each No. 1 tube was tested immediately before dilution with saline to make sure that inhibition was complete. This was found to be the case in every test except those using the smaller dose of iodides.

Identical results were obtained with potassium salts, except that potassium thiocyanate was not used.

TABLE I.—*Sodium Salts.*

	C.c. of 0·85% saline added to each tube.	Tube number.	Degree of hæmolysis.											
			NaCl. 2N, 0·8 c.c.		$NaNO_3$. 2N, 0·6 c.c.		NaBr. 2N, 0·6 c.c.		NaI. 2N, 0·5 c.c.		NaI. 2N, 0·25 c.c.		NaCNS. 2N, 0·25 c.c.	
			Amount of mixture added to 0·5 c.c. 10% red blood-cells.											
			0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10
Dilated with saline after allowing salt-serum mixture to stand 2 hours	2	1	4	4	4	4	4	4	4	4	4	4	4	4
	4	2	4	4	4	4	4	4	4	4	4	4	4	4
	8	3	3	4	3	4	3	4	2	3	3	4	3	4
Controls, Set I: Serum diluted with saline before adding salt	2	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	5	4	4	4	4	4	4	4	4	4	4	4	4
	8	6	3	4	3	4	3	4	3	4	3	4	3	4

The volume controls (Set II) are not included in the tables. The results were identical with those of the other controls (Set I), *i. e.* full complement activity was maintained except when the smaller amount (0·05 c.c.) of the highest dilution was tested against the 0·5 c.c. of 5 per cent. red blood-cells. This test involves a further tenfold dilution and a slight consequent weakening of activity was noted, indicated by the figure 3. This effect was shown by all the controls that were carried out.

Inspection of Table I shows that the inactivation of complement by neutral salts (used in the quantities stated) is completely reversed on dilution except perhaps in the case of the larger amount of iodide (0·5 c.c.). None of the quantities used, however, was very greatly in excess of the M.I.D., and it was thought possible that reversibility might not be perfectly complete if much larger doses of neutral salt were used. A second experiment was therefore carried out, using amounts of salts up to 3 and 4 times the M.I.D. The results show a marked difference between iodide and thiocyanate on the one hands, and chlorides, nitrates and bromide on the other, as is seen from Table II. The

TABLE II.—*Results Obtained as for Table I, but Using Larger Amounts of Salts and Testing against 0·5 c.c. of 5 per cent. Suspension of Red Blood-cells.*

	C.c. of 0·85% saline added to each tube.	Tube number.	Degree of hæmolysis.																	
			NaCl. 4 N, 1·0 c.c.		KCl. 4 N, 1·0 c.c.		$NaNO_3$. 2 N, 1·0 c.c.		KNO_3. 2 N, 1·0 c.c.		NaBr. 2 N, 1·0 c.c.		KBr. 2 N, 1·0 c.c.		NaI. 2 N, 1·0 c.c.		KI. 2 N, 1·0 c.c.		NaCNS. 2 N, 0·5 c.c.	
			Amount of salt-serum mixture added to 0·5 c.c. 5% red blood-cells.																	
			0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10
Saline added after allowing salt-serum to stand 2 hours	2	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0	0
	4	2	3	4	4	4	4	4	3	4	4	4	3	4	0	0	0	0	0	0
	8	3	3	4	4	4	3	4	3	4	3	4	3	4	0	0	0	0	0	0
Saline added before adding salt to serum	2	4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	4	4
	4	5	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	8	6	3	4	4	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

TABLE III.—*Effect of Salts Acting upon Serum Overnight.*

	C.c. of 0·85% saline added to each tube.	Tube number.	Degree of hæmolysis.																	
			NaCl. 4 N, 1·0 c.c.		KCl. 4 N, 1·0 c.c.		$NaNO_3$. 2 N, 1·0 c.c.		KNO_3. 2 N, 1·0 c.c.		KBr. 2 N, 1·0 c.c.		NaI. 2 N, 0·5 c.c.		NaI. 2N, 0·25 c.c.		KI. 2 N, 0·5 c.c.		KI. 2N, 0·25 c.c.		NaCNS. 2N. 0·25 c.c.	
			Amount of salt-serum mixture added to 0·5 c.c. 5% red blood-cells.																	
			0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10	0·05	0·10
Saline added after allowing salt-serum to stand 24 hours before dilution	2	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	1	1
	4	2	4	4	4	4	4	4	4	4	4	4	1	1	3	3	1	2	2	2	0	0
	8	3	4	4	3	4	3	4	3	4	3	4	0	1	2	2	0	1	2	2	0	0
Saline added before salt and then allowed to stand 24 hours	2	4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	4	4
	4	5	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	8	6	4	4	4	4	3	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4

effect of the latter salts, even in higher concentrations, is still completely reversible, whereas iodide and thiocyanate have irreversible effects.

The marked difference in the inhibitory effect of iodides and thiocyanates compared with that of bromide, chloride and nitrate was still more striking in the results of a third experiment. *Large* doses of chloride, bromide and nitrate, and *small* doses, just in excess of the minimum I.D., of iodide and thiocyanate were allowed to act upon the serum for eighteen hours, *i.e.* overnight. The small doses under these conditions now caused an inhibition which was only partially reversible. The effect of the large quantities of chloride, etc., was still, however, completely reversible in spite of the greatly prolonged time of action. These results are shown in Table III.

It will be seen from Table III that except for chlorides no results are given for salts using 1 c.c. of 4 N salt to 1 c.c. of serum. Actually such experiments were tried, but the dilutions used (4 and 8 c.c. of saline) did not reduce the final salt concentration to a point below the inhibitory concentration. This was shown by the controls, in which the complement activity was consequently affected. However, with chlorides, bromides and nitrates, there was no difference between the reversibility tests and the controls, showing that any loss in complement activity was not due to an irreversible salt effect, but to the too high concentration of salt remaining after dilution with saline.

SUMMARY.

The reversibility of the inhibition of serum complement by certain neutral salts has been studied. The effect of sodium and potassium chlorides, nitrates and bromides appears to be completely reversible on dilution even after twenty-four hours' action of large doses. Iodides and thiocyanates, however, behave differently. Small amounts (just in excess of the minimum inhibitory dose) acting for twenty-four hours, or larger doses acting for two hours, produce an inhibition that is largely or entirely irreversible on dilution.

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EXPERIMENTAL HYPERTENSION AND THE ARTERIAL LESIONS IN THE RABBIT.

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IT is well known that the aortic (depressor) and sinus nerves (*blutdruckzügler* or "*buffer*" nerves) exert in the main a tonic inhibitory influence on the circulatory centres in the spinal bulb (Hering, 1927; Koch, 1931; Heymans, 1933; Wright, 1932). Removal of all four buffer nerves in acute experiments results in a considerable rise of blood-pressure in the rabbit, cat or dog. The chronic effects produced by removing some or all of the buffer nerves have been studied by various workers. Bruns and Genner (1910) noted cardiac hypertrophy and aortic thickening in four dogs that were made to work daily on the treadmill following section of the aortic nerves. Velluda (1927), however, was unable to confirm these results, but in his experiments the forced exercise was omitted. Hirsch and Thorspecker (1912) described arteriosclerosis in the rabbit resulting from section of the aortic nerves followed by repeated injections of adrenaline; similar injections in animals with intact aortic nerves were without effect. Koch and Mies (1927, 1929) extirpated the aortic and sinus nerves in the rabbit in two stages separated by an interval of 10 to 14 days. The animals were allowed to survive for periods ranging from 14 to 511 days. In the case of 16 animals the blood-pressure was determined terminally by inserting a cannula into the carotid artery in the unanæsthetized tracheotomized animal. In 9 of these, pressures over 150 mm. were obtained. The normal average pressure in the rabbit was taken to be 100 mm. In 9 animals repeated determinations were made under anæsthesia by a bloodless method (Koch and Mies, 1928), and in 7 of these pressures over 140 mm. were recorded. In the case of the operated animals in which the blood-pressure was not found to be chronically raised, section of the vagi in acute experiment caused a marked elevation. It was suggested that when failure to produce hypertension occurred it was due to many of the aortic fibres running in the vagus nerve instead of separately, and thus escaping division. The chief pathological changes noted in these animals by Nordmann (1929) were in the aorta, in the heart and the kidney. The aortic lesion consisted of medial degeneration with secondary calcification. Diffuse or discrete patches of fibrosis were observed in the heart, and a certain degree of destructive change was seen in the glomeruli of the kidney. Sclerosis was noted at times in the pulmonary arteries. The extent of the aortic lesion was thought to be proportional to the degree of hypertension which developed. Mies (1929) has observed a chronic elevation of the blood-pressure in the rabbit following denervation of the carotid sinuses alone. Koch (1921) and also Heymans (1931) report the production by similar methods of a persistent hypertension in the dog.

METHODS.

In these experiments rabbits were exclusively employed. The removal of the buffer nerves was carried out in two stages with an interval of about one month. Owing to the difficulty of identifying the aortic nerve with certainty even in the rabbit, and the undeniable fact that a certain number of aortic fibres run in the vagus trunk, it was decided to remove the vagus nerve together with the aortic branch on the right side, and the aortic nerve alone on the left side where it is larger and more readily recognized. Bilateral section of the vagi in the neck is followed by death from asphyxia because of laryngeal obstruction, and could therefore not be employed. In many experiments the cervical sympathetic chain was also removed, as some of the aortic fibres often run in close association with it.

It was felt that the methods of recording blood-pressure employed by previous workers were open to criticism. The observations of Koch were made (as described) either bloodlessly on anæsthetized animals, or else by inserting a cannula into the artery of an unanæsthetized animal. In both cases serious adventitious factors come into operation which may invalidate the result. It was decided to prepare carotid loops according to the method of van Leersum (1911), which enable the blood-pressure to be measured painlessly for an indefinite period.

The detailed procedure was as follows :

Anæsthesia.—The anæsthetic employed was usually nembutal given intraperitoneally or intramuscularly in doses of 0·035 gm. per kg. body-weight, followed by a little ether when necessary. The animals usually recovered consciousness completely within 2 to 3 hours, and suffered no ill after-effects. Larger doses of nembutal in our experience result in a high proportion of fatalities during the operation or soon after.

Skin preparation.—When the animal was unconscious the hair over the anterior surface of the neck was widely clipped. A paste of barium sulphide, starch and zinc oxide was then spread over the area and allowed to remain on for 5 minutes ; it was then scraped off with a very blunt scalpel blade, and it was usually found that satisfactory depilation was thus effected. The skin was washed with water, soap and water, and finally spirit, and allowed to dry. No other antiseptic was applied, as this was found to increase the number of failures when carotid loops were being prepared.

First stage operation : Removal of buffers (right side).—A longitudinal incision is made a little to the left of the midline extending from below the chin to just above the sternum. The muscles are incised about $\frac{1}{4}$ in. to the right of trachea and the common carotid artery is exposed. The internal carotid artery is dissected out and tied with silk thread in two places—just at its origin, and about $\frac{1}{4}$ in. more distally—and divided. The sinus caroticus is thus mainly removed. The common and external carotids are carefully cleaned from all the related structures to ensure that no nervous connections persist from any remnant of the sinus that may be left. The right vagus and aortic nerves (and often the sympathetic chain) are identified, and a segment 1 in. long removed from them below the level of origin of the superior laryngeal nerve.

Preparation of carotid loop.—The common carotid is now cleaned along the whole length of the incision ; a thyroid branch has frequently to be tied

and cut. The artery is gently pulled forward, and the divided muscles are stitched with catgut behind the vessel. A second skin incision is now made parallel to the first and about an inch to the right. The band of skin thus marked out is carefully lifted from the underlying structures without injuring it, wrapped round the artery and stitched in the form of a tube. The free skin edges in the neck are then stitched together behind the loop; little difficulty is usually experienced in drawing them together. The corners are stitched as in a tubular skin-graft, so that no bare skin is exposed. All the skin stitching is carried out with silk, which need not be removed. Interrupted sutures are used throughout.

Second stage operation (*left side*).—The skin incision is made ½ in. to the left of the loop. The carotid sinus is exsected and denervated as before. The aortic nerve is isolated and removed, sometimes together with the sympathetic trunk. The vagus nerve was always left intact.

The Normal Blood-pressure in the Rabbit.

In any study of hypertension it is, of course, essential to know the normal blood-pressure range for the species. According to Tigerstedt (1922) the mean systolic blood-pressure in the rabbit is 100 mm., with a range of 80 to 120 mm. Koch and Koller (1932) analyzed the readings obtained in 63 animals in the laboratory at Cologne under a variety of acute conditions and under different forms of anæsthesia. The results fall along a very smooth distribution curve, with a mean pressure of 95 mm. (see Fig. 3 A), and a range of 65 to 125 mm. Van Leersum (1911), using the carotid loop method, gives the normal range as 70 to 140 mm. Scarff (1927), using the same method, found an average pressure of 95 mm. Tregubow (1928) and Cohn and Loewy (1920) obtained maximal readings of 115 mm. The only discordant opinion in the literature is that of Dominguez (1927), who made an enormous number of readings over long periods of time on more than 90 untreated animals and obtained a pressure range of 80 to 170 mm. He would only regard as evidence of hypertension a pressure fluctuating persistently over 180 mm. But his method of determining blood-pressure is open to serious question. He takes a series of readings on an animal at one sitting and regards each of these as significant, whatever be the state of the animal. His range, therefore, applies to all states of physiological activity and is useless for comparative purposes. The essence of blood-pressure readings is that they should be taken under standard and comparable conditions, and it is usually agreed that these should be basal or resting conditions. When the blood-pressure is taken in man and evidence of emotional stress is present, the reading is ignored and repeated when the patient has calmed down. For these reasons we cannot agree with the criteria of Dominguez. We regard the blood-pressure as the lowest reading that can be obtained under resting conditions, and so have adopted the range given by the other workers. We regard the average resting blood-pressure in the rabbit as about 100 mm.

Technique of Blood-pressure Reading.

We find that the rabbits soon become accustomed to having their blood-pressure read. This is done by means of a small rubber bag encased in canvas which is wrapped round the loop and clipped. This bag is filled with water, and

communicates with a bulb filled with mercury. The level of the mercury in this bulb can be altered by means of an adjustable reservoir, and so the pressure in the bag can be raised or lowered. By a side tube the bulb communicates with a mercury manometer and so the pressure in the bag can be read directly.

The systolic blood-pressure alone is recorded by means of the palpatory method. A finger is placed on the loop above the obliterating bag. The carotid pulsations are easily felt, and the pressure is raised till the pulsations disappear. The point of disappearance is quite sharp.

After the first 4–5 days the rabbits remain quite still during the determination. Six to eight readings are taken at each determination, and it is found that the last four are in very close agreement. Should the rabbit become excited we find it very difficult to determine the blood-pressure, because the pulse-rate becomes fast and a diffuse end-point is obtained when for mechanical reasons the reading is often much lower than it should be. As an additional precaution the blood-pressure is measured at approximately the same time of day and the palpation is carried out by the same person. It is quite impossible for accurate readings to be taken without assistance. In all cases the readings adopted were well on the conservative side.

RESULTS.

A. *Blood-pressure Changes.*

1. *Unilateral operations.*—The total number of animals used in the blood-pressure work was 57. In the case of 32 of these satisfactory carotid loops were made. The proportion of successful results (56 per cent.) is low, because in addition to the normal risks and difficulties associated with preparing the loop there was the additional danger of the unilateral denervation ; the latter operation by itself is associated with a fair mortality-rate. Seven of the animals either tore their loops through or died of infection before more than a few determinations had been made. The remaining 25 animals (with one exception) had their blood-pressure studied during the interval between the first and second operation (usually about 4 weeks). Satisfactory readings cannot be obtained till 2 weeks after the first operation, as time has to be given for the loop to heal. In the case of one animal (No. 153) blood-pressure readings were taken over a period of 9 months. The animal still survives.

Fig. 1 summarizes the results. The ordinate represents the percentage distribution of the blood-pressure readings which are shown along the abscissa. The average blood-pressure is about 115 mm. ; 80 per cent. of the readings fall between 105 and 120 mm. The distribution curves are very smooth, and comparable for the whole series and for No. 153 alone.

These results indicate that unilateral denervation gives rise to a slight persistent elevation of the blood-pressure. These figures also serve as a valuable control to those obtained after bilateral denervation.

2. *Bilateral operations.*—In 24 animals with loops, denervation was carried out on the second side ; 15 of these did not survive the second operation, either dying immediately or after a short interval of heart failure, sometimes with pulmonary œdema. In 4 others the loop was injured or the neck became infected. Five animals survived in a satisfactory state. The details are as follows :

Number.	Date of second operation.	Fate.
97	26.x.31	Killed 4.ix.32 (canker of ear).
136	27.i.32	Died 9.vi.32 of attempted adrenalectomy.
137	13.i.32	Killed 30.ix.32.
146	20.i.32	,, 5.iii.33.
148	21.i.32	,, 30.ix.32.

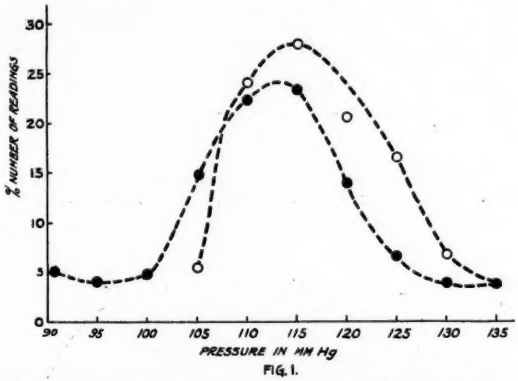


FIG. 1.

FIG. 1.—Distribution of blood-pressure readings in rabbits subjected to unilateral operation. (Removal of carotid sinus, right vagus and aortic nerves, and sometimes right cervical sympathetic.) Ordinate: Number of readings expressed as percentage of total readings. Abscissa: Systolic blood-pressure (mm. Hg.). Black dots: All observations (180 readings). Clear circles: Readings on Rabbit 153 (50 readings).

The blood-pressure results are indicated in Figs. 2, 3 and 4. Fig. 2 shows the distribution curves of each of the 5 animals that had the bilateral denervation. The curves are comparatively smooth and similar. Fig. 3 shows the distribution curve obtained from pooling the results of the whole series, and contrasts it with the curves in the unilateral and unoperated series. The entire range was 120 to 190 mm. The average blood-pressure in this series was 160 mm. In nearly 80 per cent. of cases the range was between 150 and 190. The results presented seem to indicate convincingly that a considerable hypertension is produced by the double denervation.

The hypertension did not develop to its full extent till three to four weeks after the second operation. The blood-pressure level observed over long periods showed considerable fluctuations as indicated in Fig. 4, which shows the course of events in animal No. 97. But it can be readily seen that the majority of the readings are higher than the range obtained after the unilateral operation.

In Rabbit 146 the left suprarenal gland was removed and the right gland denervated by cutting the right splanchnic nerve, to determine to what extent the hypertension could be attributed to an excessive outpouring of adrenaline. When the animal recovered from this operation the blood-pressure readings were of the same order as previously.

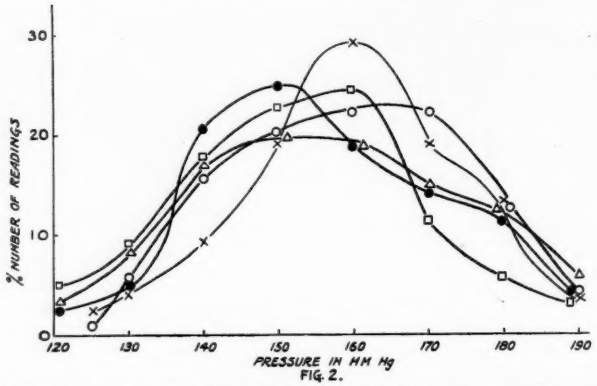


FIG. 2.—Distribution curve of blood-pressure readings in 5 rabbits subjected to bilateral denervation. ×, No. 148; O, No. 146; □, No. 137; ●, No. 136; △, No. 97.

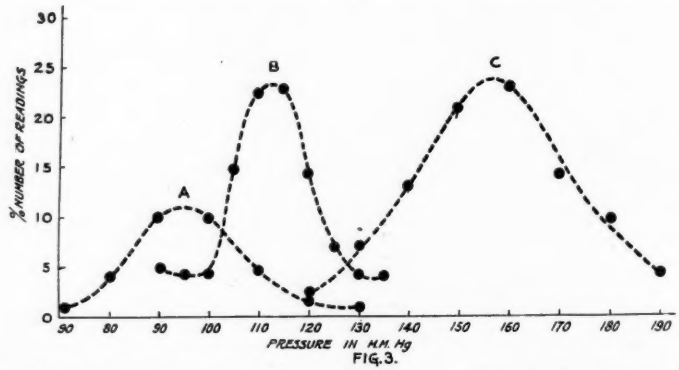


FIG. 3.—Distribution curve of blood-pressure readings in rabbits. A: Koch's normal series. B: Unilateral series. C: Bilateral series.

Blood nitrogen determinations (by the Courtauld Institute of Biochemistry) were made in all the hypertensive animals and in 3 control animals with the following results:

Hypertensive animal number.	Blood-urea.	Non-protein N.
97 (after 8 months)	45	42
137 (,, 4 ,,)	29	41
136 (,, $4\frac{1}{2}$,,)	38	40
146 (,, 5 ,,)	38	40
148 (,, $4\frac{1}{2}$,,)	30	41
Control animal number.		
54	50	44
A	53	49
149	54	44

No deviation in these values from normal was observed.

B. *Pathological Changes.*

The material investigated was obtained from 42 animals, of which 22 were killed at various times after the double operation, 12 after the single operation, and 8 were normal control rabbits from the same source of supply. A brief description of the changes found after the double operation is given first.

Aorta.—The most striking lesions were found in the aorta, and in most cases were recognizable on naked-eye examination ; they may be arbitrarily divided into three stages :

(1) The earliest lesion apparent to the naked eye consisted of a minute bleb slightly raised above the inner surface of the vessel.

(2) The next stage appeared as a thickened whitish area in the vessel-wall.

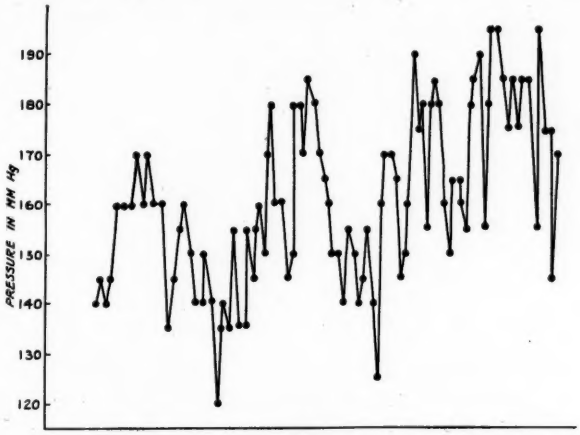


FIG. 4.

FIG. 4.—Actual blood-pressure readings in bilaterally operated Rabbit No. 97.

(3) Gross lesions were thick white calcified plaques which maintain the curve of the vessel-wall (Fig. 8).

The lesions were found most frequently in the ascending portion of the vessel and progressively less frequently towards its termination.

On microscopical examination the changes are seen to be confined to the media, and may be divided into three stages roughly corresponding with those seen by the naked eye :

(1) Early lesions show small foci of degeneration in the media, the muscle-fibres being replaced by hyaline material ; the elastic fibres appear smaller, but are otherwise unaffected. There are also present in the degenerate areas cells with palely staining finely granular nuclei and ill-defined cell outline. These cells are either degenerate muscle-cells or histiocytes ; their appearance, and also the fact that they increase in number as the lesion progresses, favour the view that they are histiocytes (Figs. 5 and 6).

(2) This stage is characterized by fragmentation of the elastic fibres, increase in the mononuclear cells and early calcification, the calcification being represented by a few acicular bodies staining deeply with hæmatoxylin (Fig. 7).

(3) Advanced lesions show a central structureless calcified area, round which is a zone showing the degenerative changes described above (Figs. 9 and 10).

The intima shows no gross changes, except in those instances in which the medial lesion is situated immediately beneath this coat, when there is some fibrosis.

The findings in the aorta are summarized :

	A. Double operation.	B. Single operation.	C. Controls.
Total number of animals	22	12	8
Marked lesion	12	No comparable changes observed in any of these animals.	
Few lesions	6		
No lesions	4		

The survival times of the animals in the bilaterally operated series were as follows :

	Marked lesions.	Slight lesions.	No lesions.
Under 10 weeks	2	5	1
		(1, 5, 6, 6, 8 weeks)	(7 weeks)
10–20 weeks	5	1	2
		(19 weeks)	(12, 15 weeks)
20–30 ,,	2	..	..
30 weeks and over	3	..	1
	(35, 48, 60 weeks)		(34 weeks)

Broadly speaking, there appears to be some relationship between the duration of the survival period and the extent of the lesions. Thus of the 8 animals that survived less than 10 weeks, only 2 had marked lesions, while of the 14 that survived longer, 10 had marked lesions. One animal (No. 137) that had been shown to have persistent hypertension during life and survived 34 weeks showed no lesions whatever. It appears, therefore, that these aortic lesions do not invariably follow the operation, even with prolonged survival with hypertension.

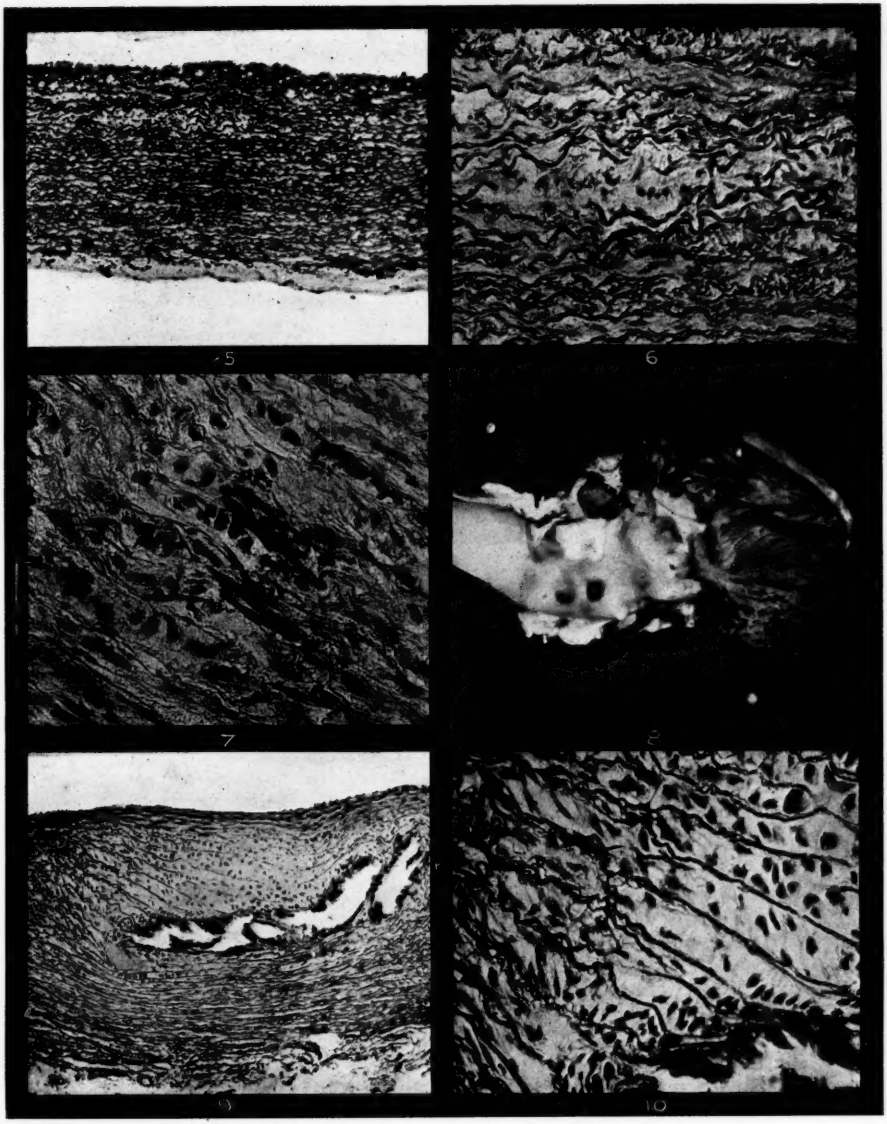
Heart.—The heart was sectioned in 12 animals killed after the double operation and in 6 after the single operation. The hearts of those rabbits which had undergone the double operation all showed a patchy fibrosis, which was most marked in the wall of the left ventricle.

Both to the naked eye and microscopically there appeared to be moderate hypertrophy of the left ventricle, but the observations on the present series are insufficient to establish definite conclusions ; a further series is being studied from the point of view of cardiovascular hypertrophy and it is hoped to publish the results of this investigation shortly.

Other organs.—Sections were taken of other organs of several of the animals, mainly those allowed to survive for a long time after the operation ; these included the kidneys, suprarenals, liver, spleen and striped muscle. Apart from the question of vascular hypertrophy, on which no definite conclusions

DESCRIPTIONS OF FIGURES.

FIG. 5.—Early lesion showing degeneration of muscle-fibres. × 90.
FIG. 6.—Early lesion showing degeneration of muscle-fibres. × 350.
FIG. 7.—Early calcification of lesion. × 350.
FIG. 8.—Advanced lesion.
FIG. 9.—Advanced lesion with marked calcification. × 90.
FIG. 10.—Advanced lesion with marked calcification. × 350.



Kremer, Wright and Scarff.

have been reached, no significant changes were found. The renal lesions described by Goormagtigh (1931) as occurring in this condition were not encountered.

DISCUSSION.

The results described in this paper indicate that unilateral destruction of the buffer nerves produces a slight rise of blood-pressure, while bilateral denervation gives rise to a marked and persistent hypertension (confirmatory of Koch and Mies). The double operation is followed in a large proportion of cases by definite changes in the aorta and in the heart. Comparable changes are not found after the unilateral operation or in control animals.

In criticism of work carried out on experimental vascular disease in the rabbit, it is often pointed out that "the rabbit is notoriously prone to arterial disease". If by this is meant that spontaneous lesions are commonly met with in healthy adult rabbits, the view is negatived by a consideration of the literature (*e. g.* Bennecke, 1908 ; Dominguez, 1928), and our own considerable experience derived from this and previous studies. Lesions can undoubtedly be induced more readily in the rabbit by means of appropriate experimental conditions than in other species. This, however, merely indicates that it is a suitable animal for such studies, in the same way that one selects an animal which is readily liable to scurvy for a study of this condition. In these experiments vascular lesions were not observed in any of the control or unilaterally denervated animals, or even in a proportion of the bilaterally operated animals. This fact makes the positive lesions observed in a proportion of the bilaterally operated animals all the more significant.

The hypertension can be attributed to loss of the normal afferent inhibitory control. The marked fluctuations which occur spontaneously in these animals are not unexpected, in view of the fact that the normal blood-pressure controlling mechanism has been removed. The presence of adrenaline secretion is apparently not essential for the persistence of the hypertension.

Though there appears to be a causal relationship between the operative procedure and the production of the cardio-vascular lesions, there is as yet no evidence to indicate how the latter are produced. The most obvious suggestion is that they result from the abnormal strain thrown on the heart and vessels by the hypertension ; but, as has been pointed out, there is not an invariable relationship between the presence of hypertension and the production of the lesions.

SUMMARY.

1. The aortic and sinus nerves (buffer nerves) have been extirpated in rabbits that were allowed to survive for long periods. The blood-pressure changes were recorded by means of the carotid loop method.

2. Following unilateral denervation there is an elevation of the blood-pressure from the normal level (about 95 mm.) to about 115 mm. Following bilateral denervation there is a persistent marked hypertension, the average blood-pressure being 160 mm.

3. Degenerative changes in the aorta and fibrosis of the heart may take place in a high proportion of cases following the bilateral denervation. These changes are not observed in control animals or after unilateral operation.

4. The mode of production of the lesions is uncertain, but they are not the invariable accompaniment of hypertension.

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A STUDY OF THE ENZYMES OF THE AGALACTIA VIRUS.

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THE presence of a virus in culture can be detected by its pathogenicity or by ultra-microscopic methods; or by inclusion bodies in the tissue-cells. A saprophytic virus such as that described by Barnard (British Association, 1931) or a virus which has lost its pathogenicity in culture can similarly be detected ultra-microscopically, or by the production of turbidty or measurable chemical changes in the medium. The metabolic processes of viruses should give a further clue to the nature of these small organisms as well as a method of detecting their presence.

The only two filter-passing organisms which are known to be capable of growth in the absence of living cells (except the saprophytes referred to) are the viruses of bovine pleuro-pneumonia and of Agalactia, a disease of the mammary glands of goats. These two small organisms seemed, therefore, the most suitable for an investigation of virus metabolism, and it was hoped that their chemical behaviour in culture might give a clue to the general lack of success in the cultivation of the other pathogenic viruses.

The virus of Agalactia grows and retains its pathogenicity in Hartley's broth with 5–10 per cent. horse-serum added to it (Galloway, 1932). Unlike the virus of bovine pleuro-pneumonia, which readily produces acid in such a medium, no obvious chemical change occurs during the growth of Agalactia,

and no chemical activities seem to have been described for it hitherto. The medium becomes opalescent in 3–4 days (occasionally in 24 or 48 hours), and then develops a thin fragile scum on the surface. The pH of the medium does not change during growth.

We had previously found that growth of the virus of bovine pleuro-pneumonia could be detected and roughly estimated by a determination of the lactic dehydrogenase activity of the culture, using the standard methylene-blue technique (Holmes and Pirie, 1932). Since most strains of pleuro-pneumonia become non-pathogenic very rapidly in culture, this is the most convenient method available for following the multiplication of the organism. We applied this method to Agalactia, and found that this virus also contains dehydrogenases. Uninoculated medium only reduces methylene-blue after many hours' incubation at 37° C., but during the growth of Agalactia, as judged by the increase in the turbidity of the medium, the reducing power of the culture increases gradually, and usually reaches a maximum the day after the scum first appears on the surface. Two c.c. of a four-day-old culture usually reduces 0·2 c.c. of 1/3000 methylene-blue in a Thunberg tube in between half and one hour at 37° C. The reducing power of a culture of Agalactia is very much less than that of a culture of bovine pleuro-pneumonia, an average reduction time for 2 c.c. of this virus culture being 5–10 minutes with 0·2 c.c. of methylene-blue. Unknown conditions of the medium sometimes cause very rapid growth of Agalactia, and these cultures may possess as active dehydrogenases as those of pleuro-pneumonia.

It is possible that the greater activity of these quick-growing cultures is due to the presence of a larger number of living organisms, and that the more usual cultures contain relatively few at any one moment. The dehydrogenase activity of such cultures does not seem great enough to provide energy for the growth of any large number of organisms.

The addition of substrates to a serum-broth culture of Agalactia does not increase the rate of reduction of methylene-blue; the substrates already present in the broth presumably saturate the enzymes of the virus. In order to determine what substances are oxidized by Agalactia, it was therefore necessary to obtain it free from an excess of serum broth. We found that by centrifuging a culture for one hour at 3500 r.p.m. a small deposit was obtained. If this was drained and suspended in saline, the suspension was able to reduce methylene-blue in the presence of certain substrates and contained active virus, as judged by sub-cultivation tests. Such a suspension of virus in saline reduces methylene-blue in the presence of broth, serum or lactate quite regularly and definitely. In the absence of these substrates reduction is slow, if all traces of the original medium have been removed by draining. Many other substrates, such as galactose, lactose, glucose, glutamate, formate, aspartate, succinate and glycerol have been used, but only equivocal results have been obtained. Slight reduction occurred at some times, but none at others. It seems as if the virus contains very small amounts of several dehydrogenases, but that most of these enzymes are only demonstrable when the virus is exceedingly concentrated. Reduction of methylene-blue by the virus in the presence of broth, serum or lactate can be shown regularly.

Early irregularities in the results were later explained by the fact that the activity of the broth and lactic dehydrogenases can disappear quite rapidly

TABLE I.—*Activity of Dehydrogenases.*

Whole culture.	Spun down virus.			
	Saline.	Lactate.	Broth.	Serum.
..	∞	110	75	..
23	∞	..	..	34
42	∞	..	11	21
54	∞	39	23	..
..	∞	60	..	..

·2 c.c. of 1/3000 methylene-blue plus 2 c.c. of whole culture or plus 1·0 c.c. of spun-down virus in saline and 1·0 c.c. of substrate were used per test. The figures are reduction times in minutes.

in a saline suspension of virus at room temperature. Half an hour's gap between tests may make an appreciable difference to the time of reduction of methylene-blue.

Since bacterial dehydrogenases are in general known to be much more resistant, we have investigated the fragility of the enzymes of Agalactia and the relation between the loss of enzyme activity and viability.

A serum-broth culture was centrifuged for one hour at 3500 r.p.m. in corked sterile tubes. The supernatant fluid was then poured off, and the sediment was drained by standing the tube upside down on filter-paper. A few c.c. of sterile saline were added to the deposit, and the suspension of virus was transferred to a sterile test-tube and its volume made up to 1/10 that of the original culture. The enzyme activity and the viability of this suspension were then tested at once and after various treatments.

Since the enzyme activity of the suspension of virus diminished in an open tube at room temperature, it seemed possible that oxygen was the destructive agent. In order to test this the effect of nitrogen and oxygen on the virus was compared. Three c.c. of the suspension were pipetted into a sterile tube, which had a sterile tightly-plugged capillary pipette passing through its own plug and reaching to the bottom. The tube was clamped in a beaker of water at 37° C., and the compressed air or nitrogen tube was attached to the capillary pipette. A fairly vigorous stream of gas was then passed through the suspension of virus. After a certain time aëration was stopped, and one drop or one loopful of the suspension was introduced into a tube containing 5 c.c. broth plus 0·5 c.c. of horse-serum to test viability. The rest of the suspension was used for the estimation of dehydrogenase activity. In some experiments a final concentration of 1/5000 HCN was added to the suspension before bubbling with air in order to see if cyanide had any preservative effect.

The enzymes of the virus seem to be equally destroyed by air or nitrogen, or by air in the presence of cyanide, so that there is no ground for the assumption that the dehydrogenases are destroyed by oxidation. Since, however, traces of oxygen remain in the suspension at the beginning of treatment with nitrogen, it cannot be said definitely that destruction does occur in the complete absence of oxygen. The nitrogen was obtained from cylinders, and was passed through pyrogallol and water before use.

At the same time as the enzymes are destroyed so also growth is impaired. In those cases where the change in enzyme activity is greatest, so the delay in the growth of the aërated virus is also greatest. It has been found that a five-hundred-fold dilution of a culture before inoculation only causes a delay

TABLE II.—*Fragility of Enzymes.*

Time of bubbling in minutes.	Gas.	Reduction. time.	Growth time.	Remarks.
..	..	65 min.	2 days	Drop inocns.
40	O_2	∞	No growth	,,
40	N_2	∞	,,	,,
40	O_2 + HCN	∞	No culture	..
..	..	48 min.	3 days	Loop inocns.
40	O_2	8 hours	8 ,,	,,
..	..	28 min.	11 ,,	,,
30	O_2	115 ,,	10 ,,	,,
..	..	34 ,,	5 ,,	,,
15	O_2	8 hours	8 ,,	,,
15	N_2	8 ,,	No culture	..
15	O_2 + HCN	8 ,,	,,	..
..	..	20 min.	2 days	Drop inocns.
60	O_2 + HCN	7 hours	4 ,,	,,
60	O_2	7 ,,	3 ,,	,,
..	..	27 min.	2 ,,	,,
40	O_2 + HCN	∞	4 ,,	,,

1 c.c. of virus suspension plus 1 c.c. of broth plus 0·2 c.c. of 1/3000 methylene-blue were used in the reduction tests.

of 48 hours in the appearance of growth, whereas a twenty-fold dilution of a culture shows only a slight reducing power towards methylene-blue. Growth on subcultivation is a more delicate test for the presence of Agalactia than capacity to reduce methylene-blue. The long delays in the appearance of growth after treatment of a virus suspension with air or nitrogen show that a great part of the virus is destroyed by this method.

Since the fragility of the enzymes of Agalactia is much greater than knowledge of bacterial enzymes would lead one to suspect, it is possible that the difficulty in demonstrating enzymes in the smaller viruses may be due to their very great instability. In the preparation of virus filtrates a considerable time is usually taken, and the virus must receive a certain amount of mechanical violence during filtration. If a suspension of Agalactia is aërated in the presence of broth no destruction of the enzymes occurs, and there is the possibility that the effect of broth in promoting the filterability of some viruses is due to an action of this sort.

A suspension of Agalactia in saline will take up oxygen in the presence of broth or lactate solutions. This oxygen uptake was measured in a differential Barcroft manometer. The respiratory enzymes are also destroyed by shaking the suspension of virus in air at 37° C. for a short time. In certain cases they seemed to be more resistant than the corresponding dehydrogenases, but as the figure shows, the destruction is greater the longer the virus is shaken, and the viability, dehydrogenases and aërobic enzymes seem to diminish in a roughly parallel way (Fig. 1). The uptake of oxygen is much greater than the corresponding methylene-blue reduction, and is probably the source of energy for the growth of the virus. Since Agalactia will grow under strictly anaërobic

conditions in a McIntosh and Fildes anaërobic jar, it was unexpected to find such a large oxidative metabolism. After growth anaërobically the activity of the dehydrogenases is not greater than after aërobic growth.

Perdeau and Todd (1933) found that very small amounts of methylene-blue inactivated several phages and many viruses in the presence of light and air. Pathogenicity was the only test available for the presence or absence of the viruses after exposure to the dye. It seems quite possible that methylene-blue might destroy pathogenicity without affecting viability in culture, but this cannot be tested on the viruses which are unable to grow in lifeless media.

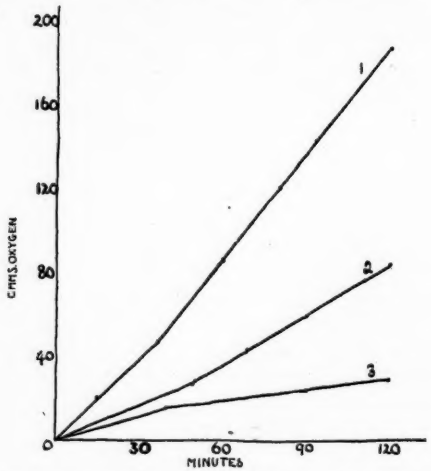

FIG. 1.—Oxygen uptake of centrifuged Agalactia. 2·0 c.c. of saline suspension of virus and 1·0 c.c. of serum broth per cup. 1. Unshaken virus. 2. Virus shaken in air for 30 minutes. 3. Virus shaken in air for 60 minutes.

In the case of Agalactia, the effect of exposure to light in the presence of methylene blue on the viability in serum broth and on the activity of the dehydrogenases can be tested more readily than the effect on pathogenicity, since Agalactia is only pathogenic in goats and sheep. Therefore by the use of this virus the question whether methylene-blue destroys pathogenicity only or viability also can be partly answered. The experimental method of Perdrau and Todd has been followed almost exactly. Ten c.c. of culture plus ·25 c.c. of 1/3000 methylene-blue has been exposed to a 200-watt lamp at a distance of 20 cm. The culture was kept cold by the interposition of a layer of running tap-water between it and the light. After exposure a drop of culture was inoculated into a tube containing 5 c.c. of broth plus ·5 c.c. of serum, and 2 c.c. of culture were used to determine the activity of the dehydrogenases. Under these conditions we found that short exposures of Agalactia to light in the presence of methylene-blue resulted in an apparently parallel loss in enzyme activity and viability of the virus. Exposure of the culture to light in the absence of the dye causes a slight slowing of the reduction-time. This is apparently due to a reduction in the substrate concentration rather than to a

destruction of the enzymes themselves, since addition of broth to such an exposed culture restores its capacity to reduce methylene-blue to the original level. The loss of reducing power after exposure in the presence of methylene-blue is very much greater, and cannot be restored by the addition of broth. Complete destruction of the virus was only attained in one experiment, while the dehydrogenase activity was usually reduced to a non-detectable level after one hour's exposure. This is not incompatible with the view that viability and enzyme activity are destroyed in a parallel way by exposure to the dye, since, as we have already shown, growth on subcultivation is a more sensitive test for the presence of Agalactia than capacity to reduce methylene-blue.

TABLE III.—*Effect of Methylene-blue.*

Time of exposure.	Reduction time in minutes.			Growth time in days.		
	Control.	Exposed control.	Exposed + dye.	Control.	Exposed control.	Dye.
1 hour	66	80	∞	3	4	10
1 ,,	48	65	∞	7*	10*	None.*
30 min.	57	75	152	3	3	4
1 ,,	30	30	35	3	3	3
5 ,,	..	27	50	..	3	4
10 ,,	..	31	87	..	3	4
20 ,,	..	41	180	..	3	4

Concentration of dye during exposure to lamp: 1/120,000. 2 c.c. culture + ·2 c.c. dye (total) used per test of reducing capacity. Turbidity of medium and surface scum were taken as signs of growth.

* Loop inoculations were used.

It was thought at first that the thin scum that forms on the surface of a culture of Agalactia might be a lipoid (in its unpurified state it did contain phosphorus), and it was therefore decided to investigate the phosphorus distribution before and after growth of the virus. The phosphorus content of the acid-soluble and the lipoid fractions were investigated. Changes in the inorganic phosphorus during growth could not be detected, as the fraction was too large to allow the detection of any small difference. The method of Eggleton and Eggleton (1929) was followed in the estimation of acid-soluble phosphorus. This fraction rose very slightly during growth, but so slightly that no great importance can be attached to the results. To estimate the lipoid phosphorus a 3 c.c. sample was run into 20 c.c. of a 3/1 mixture of alcohol and ether and the precipitate was extracted by boiling. A total volume of 50 c.c. of alcohol-ether was used for extraction. The filtrate was evaporated to dryness and re-extracted with 20 c.c. of petroleum ether. The evaporated petroleum ether-extract was incinerated with sulphuric acid and perhydrol and estimated in the usual way (Eggleton and Eggleton, 1929).

The values for lipoid phosphorus were small, but reasonably good duplicates could be obtained by the method described. A small but definite drop in the lipoid phosphorus was nearly always found on comparing a culture after growth with a tube of uninoculated medium, which had been incubated for the same length of time or with the same culture before growth began.

We have also investigated the nitrogen metabolism of Agalactia. Non-protein nitrogen, amino-nitrogen and ammonia were estimated in cultures

TABLE IV.—*Lipoid Phosphorus of* 3 *c.c. Culture.*

Uninoculated medium.	Incubated medium.	Virus.
·166	..	·123
·032	·043	·014
·071	·060	·072
..	·020	·013
·044	·026	·016
·012	..	·005
·039	·039	·019

before and after growth, and in uninoculated tubes of medium which were incubated with the culture. The presence of fresh serum in the medium makes this control a necessary one. No change was found in any of these values after growth of the virus. If the virus obtained the energy for its growth from breakdown of nitrogen compounds, a change in the non-protein, amino or ammonia nitrogen should have been detectable, judging by the magnitude of the oxygen uptake. Change in the nitrogen partition due to synthesis of the proteins of the virus would be too small for estimation.

SUMMARY.

The virus of Agalactia, when centrifuged out of a culture, can be shown to reduce methylene-blue in the presence of lactate, broth or serum.

The growth of the virus in culture can be demonstrated by the increased rate of reduction of methylene-blue by a measured amount of culture. A culture of Agalactia shows a large oxygen uptake, and so also does a saline suspension of virus in the presence of lactate or of broth. The dehydrogenase and oxidative systems are extremely sensitive to mechanical agitation. A saline suspension of virus loses all its reducing capacity after bubbling with air, or nitrogen, or air in the presence of cyanide. The number of viable organisms is also very much reduced. Broth has a protective effect on the enzymes and the viability. It is suggested that these facts may be of importance in connection with the filterability of some other viruses. The enzyme activity and the viability are very greatly reduced by exposure to light in the presence of very small amounts of methylene-blue.

No change can be shown in the nitrogen distribution of a culture during growth and no disappearance of glucose can be detected. There is nearly always a small decrease in lipoid phosphorus during the growth of a culture.

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